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*February 02, 2006*

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**APPLICATION NUMBER: 60/569,625**

**FILING DATE: May 11, 2004**

**RELATED PCT APPLICATION NUMBER: PCT/US04/29293**

**THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS US60/569,625**



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Revised PTO/SB/16 (8-00)  
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Attorney Docket No. 31978-201011

## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)					
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)			
Frank Alfredo	CUTTITTA MARTINEZ	Adamstown, Maryland Bethesda, Maryland			
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
A METHOD FOR INHIBITING ANGIOGENESIS, USING A SMALL MOLECULE					
CORRESPONDENCE ADDRESS					
Direct all correspondence to:					
<input checked="" type="checkbox"/> Customer Number		26694		→ 26694 PATENT TRADEMARK OFFICE	
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<input checked="" type="checkbox"/> Firm or Individual Name		VENABLE LLP			
Address		P.O. Box 34385			
Address					
City	Washington	State	DC	ZIP	20043-9998
Country	U.S.A.	Telephone	202.344.4000	Fax	202.344.8300
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		53		<input type="checkbox"/> CD(s), Number	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets		9		<input type="checkbox"/> Other (specify)	
<input checked="" type="checkbox"/> Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.					
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees					
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 22-0261					
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.					
FILING FEE AMOUNT (\$) 160.00					
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input type="checkbox"/> No.					
<input checked="" type="checkbox"/> Yes, the name of the U.S. Government agency is National Institutes of Health					

Respectfully submitted,

SIGNATURE

*Nancy Axelrod*

Date

5/11/04

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REGISTRATION NO.  
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44,014

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(202) 344-8334

Docket Number:

31978-201011

### USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51, and is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. SEND TO: Box Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

546829v1

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15535 U.S. PTO  
60/569625



13281 U.S. PTO

PTO/SB/17 (10-01)

Approved for use through 10/31/2002. OMB 0851-0032

U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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# FEE TRANSMITTAL for FY 2004

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT (\$) 160.00

## Complete if Known

Application Number	To Be Assigned
Filing Date	May 11, 2004
First Named Inventor	Frank CUTTITTA
Examiner Name	N/A
Group / Art Unit	N/A
Attorney Docket No.	31978-201011

## METHOD OF PAYMENT (check one)

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:

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Number

22-0281

Deposit  
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- ☒ Charge Any Additional Fee Required  
Under 37 CFR 1.16 and 1.17  
☐ Applicant claims small entity status.  
See 37 CFR 1.27

2. ☒ Payment Enclosed:

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## FEE CALCULATION

## 1. BASIC FILING FEE

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	160.00

SUBTOTAL (1)

(\$ 160.00)

## 2. EXTRA CLAIM FEES

Total Claims	Extra Claims	Fee from below	Fee Paid
0	0	0	0
0	0	0	0
0	0	0	0

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2204	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2)

(\$ 0.00)

\*\*or number previously paid, if greater; For Reissues, see above

## FEE CALCULATION (continued)

Large Entity Fee Code	Large Entity Fee (\$)	Small Entity Fee Code	Small Entity Fee (\$)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2215	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2010	2255	1005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	25403	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17 (q)	
1808	180	1808	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) \_\_\_\_\_

\*Reduced by Basic Filing Fee Paid

SUBTOTAL (3)

(\$ 0.00)

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Date

May 11, 2004

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ATTORNEYS AT LAW

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PC Docs No. 548819v1

# **A METHOD FOR INHIBITING ANGIOGENESIS, USING A SMALL MOLECULE**

## **FIELD OF THE INVENTION**

5           This invention relates generally to small molecule, non-peptide, modulators (*e.g.*, antagonists or agonists) of peptide hormones. Also described are complexes comprising such small molecules, methods of identifying the molecules as modulatory agents, and methods of diagnosis or treatment, using the molecules.

## **BACKGROUND INFORMATION**

10           Adrenomedullin (AM) is a peptide hormone implicated in the pathophysiology of important diseases such as hypertension, cancer, and diabetes. AM is a 52 amino acid peptide that belongs to the calcitonin/calcitonin gene related peptide (CGRP)/amylin/AM superfamily. In humans, this peptide is expressed by many cell types and exerts a variety  
15 of physiological roles, including vasodilatation, bronchodilatation, regulation of hormone secretion, neurotransmission, antimicrobial activities, regulation of growth, apoptosis, migration, and angiogenesis, among others.

          These activities are mediated by a complex receptor system encompassing a seven transmembrane domain polypeptide known as calcitonin receptor-like receptor (CRLR), a  
20 single transmembrane domain protein, termed receptor activity modifying protein (RAMP), and the intracellular receptor component protein (RCP). RCP is necessary for the initiation of the signal transduction pathway. Three RAMPs have been identified in mammals and their coexpression with CRLR results in different binding affinities, with RAMP1 producing a characteristic CGRP-1 response whereas coexpression of CRLR  
25 with RAMP2 or RAMP3 elicits a specific AM receptor.

          Gastrin releasing hormone (GRP) is a peptide hormone implicated in the pathophysiology of important diseases such as cancer and respiratory problems in premature babies. GRP is a 27 amino acid peptide, initially identified as the human counterpart of bombesin, a peptide found in the frog's skin. GRP has a variety of  
30 physiological roles. For example, it has antimicrobial properties, reduces food intake, and has been involved in respiratory development, and in the regulation of short-term memory, among others.

Several types of antagonists have been proposed for peptide hormones, including monoclonal antibodies and inhibitory peptide fragments, such as AM(22-52), AM(16-31), AM(11-26), and proAM(153-185). While these molecules are effective as research tools, they sometimes exhibit significant limitations as pharmaceutical agents, *e.g.*, because of the lack of humanized blocking antibodies and the short biological half-life of fragmentary peptides. There is a need for additional agents that modulate activities of peptide hormones, in particular AM and GRP, and that can be used to treat disease conditions mediated by the peptide hormones, such as the conditions noted above. Small molecule, non-peptide agents would be particularly desirable.

### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** illustrates primary screen (step #1 of the method) using a blocking monoclonal antibody. **Figure 1A** shows a schematic representation of the primary screening process. **Figure 1B** shows a photograph of part of a fully developed AM-coated 96-well plate used for the initial screening of the library. Wells A1 and A2 are not coated with AM and provide the value for non-specific background. Wells A3 and A4 have been exposed to all the reagents but the competitors and their color value provides the maximum binding for the assay. Wells A5 and A6 have been exposed to 1.2 µg/ml unlabeled monoclonal antibody and constitute a positive-competition control. Individual small molecules from the library were assayed in duplicates in wells B and C. Wells B10 and C10 contain compound VIII (697165), one of the successful competitors. Wells A7-A12 are empty. Actual absorbance values were quantified in a plate reader.

**Figure 2** shows a secondary screen (step #2 of the method) for AM-active compounds.

This figure shows secondary screening of promising compounds by induction of intracellular cAMP levels in Rat2 cells (**Figures 2A- 2C**) and in HEK 293 cells transfected with CRLR and RAMP1 (**Figure 2D**). cAMP levels were quantified by radioimmunoassay and are represented as variations from the value of the first bar, arbitrarily expressed as 100. **Figure 2A** shows variations on intracellular cAMP levels induced by a superagonist compound (compound VIII, or 697165) and an antagonist (compound VI, or 79422) in the presence and absence of 100 nM AM. Forskolin was

added as a positive control. Asterisks represent statistical significance when compared to the untreated control (first bar) or as indicated by the horizontal bars. **Figure 2B** shows dose-dependent elevation of cAMP induced by the superagonist compound VIII in the presence of 100 nM AM. Asterisks represent statistical significance when compared to addition of AM alone (first bar). **Figure 2C** shows a comparison of the effects elicited by other members of the family of compound VIII in the presence of 100 nM AM. Asterisks represent statistical significance when compared to addition of AM alone (second bar). **Figure 2D** shows the lack of effect of several compounds in the presence of 100 nM CGRP in the activation of the CGRP receptor in HEK 293 cells. Asterisks represent statistical significance when compared to addition of CGRP alone (second bar). Bars represent mean  $\pm$  standard deviation of three independent determinations. n.s.: No significant differences; \* :  $p < 0.05$ ; \*\* :  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

**Figure 3** shows a secondary screen for GRP-active compounds. This figure shows an analysis of second messengers for compounds that interfere with GRP binding. **Figure 3A** shows a quantification of IP<sub>3</sub> levels in cell line H1299 exposed to different compounds in the presence or absence of 100 nM GRP. Bars represent mean  $\pm$  standard deviation of three independent determinations. Asterisks represent statistical significance when compared to addition of GRP alone (second bar). n.s.: No significant differences; \* :  $p < 0.05$ ; \*\* :  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . **Figure 3B** shows Ca<sup>2+</sup> response induced by 1 nM GRP in H1299 cells. **Figure 3C** shows that preincubation of H1299 cells with compound XIV (54671) for 1 min. dramatically reduces the Ca<sup>2+</sup> response elicited by 1 nM GRP.

**Figure 4** shows blood pressure regulation by AM-active compounds. This figure shows representative blood pressure recordings in hypertensive SHR (A,B) and in normotensive Lewis/ssncr (C) rats following the intravenous injection of AM antagonists (XII', or 128911; XIII', or 145425), agonists (I', or 16311), or vehicle (PBS+DMSO). Synthetic AM was added in B for comparison purposes.

**Figure 5** shows the antiangiogenic effect of a GRP antagonist. This figure shows cord formation assay in matrigel with bovine retinal microvascular endothelial cells. **Figure**

**5A** shows a negative control with no additions. **Figure 5B** shows that a complex tubular lattice is induced by 5 nM GRP. **Figure 5C** shows that the simultaneous addition of antagonist compound XV' (77427) (0.5  $\mu$ M) reduces network complexity.

**Figure 6** shows a directed *in vivo* angiogenesis assay (DIVAA). Bars represent mean  $\pm$  standard deviation of five independent determinations.

**Figure 7** shows a growth inhibition assay (MTT). Bars represent mean  $\pm$  standard deviation of eight independent determinations.

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**Figure 8** shows another growth inhibition assay (clonogenic). Bars represent mean  $\pm$  standard deviation of three independent determinations. \* :  $p < 0.05$ .

**Figure 9** shows a xenograft model in nude mice injected with cell line H1299, and treated with a small molecule inhibitor of the invention. Each point represents the mean of 10 animals. \* :  $p < 0.05$ ; \*\*\*:  $p < 0.001$ .

## DESCRIPTION OF THE INVENTION

This invention relates, *e.g.*, to agents, particularly small molecule, non-peptide, agents, that modulate activities of peptides which interact with specific receptors. In preferred embodiments, the peptides whose activities are modulated are peptide hormones, most preferably adrenomedullin (AM) or gastrin releasing peptide (GRP).

The present inventors have developed a two-step screening method to identify such modulatory agents. AM and GRP were used as exemplary peptide hormones in the screening assay; a variety of other peptide hormones can, of course, also be used. The term "modulate," as used herein, includes to increase, stimulate, augment, enhance, facilitate, or potentiate, or to decrease, inhibit, suppress, interfere with, prevent, block, etc. An agent that augments the activity of a peptide hormone is said to be an agonist (in some cases, as discussed below, a superagonist); an agent that suppresses the activity is said to be an antagonist. Both AM and GRP exhibit a variety of "activities," some of which are described elsewhere herein.

To identify modulatory compounds, a library of known small molecule, non-peptide, compounds was screened. Compounds were first identified on the basis of their ability to interfere with binding between AM or GRP and their respective blocking antibodies. Compounds identified as "positive" in this first step were further screened for their ability to influence receptor-mediated biological activities (inhibition of the induction of second messengers). Using this two-step procedure, seven compounds were identified as antagonists of AM, and three as antagonists of GRP. Surprisingly, in view of the fact that the compounds were first identified because of their ability to *inhibit* the binding of the peptide to its blocking antibody, other compounds were identified that act as agonists (*e.g.*, superagonists) of the peptides. Six superagonists were identified for AM, and one for GRP. A total of 17 modulatory agents were identified.

Among the advantages of the identified small molecule, non-peptide, modulatory agents are that the molecules are stable, especially when in an organism; are small and thus exhibit good cell permeability characteristics; and are readily synthesized, allowing for the rapid, inexpensive production of large quantities.

In one embodiment, the invention relates to a method for modulating an activity of an adrenomedullin (AM) peptide, comprising contacting the peptide with an effective amount of a compound of a formula as below:

formula I;

formula II,

formula III,

formula IV,

formula V,

formula VI,

formula VII,

formula VIII,

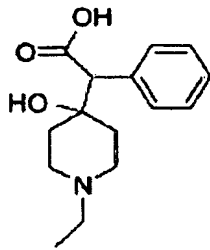
formula XII,

formula XIII,

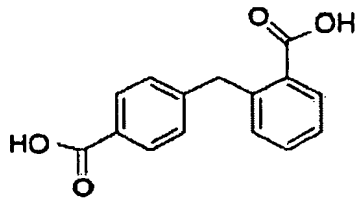
or a pharmaceutically acceptable salt thereof.

More particularly, a compound of a formula as below may be used:



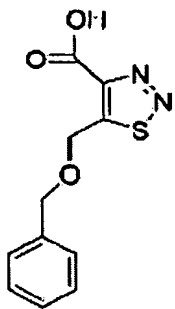


formula I',

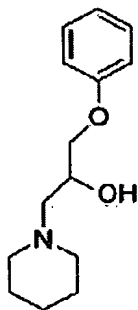


formula II',

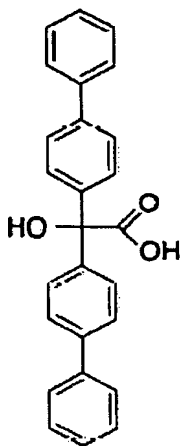
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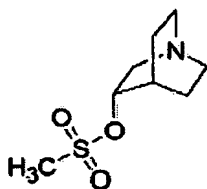
formula III',



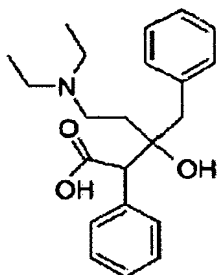
10 formula IV',



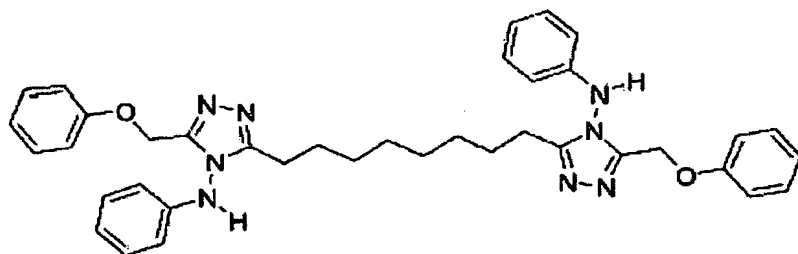
formula V',



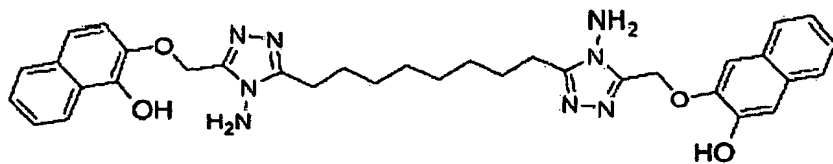
5 formula VI',



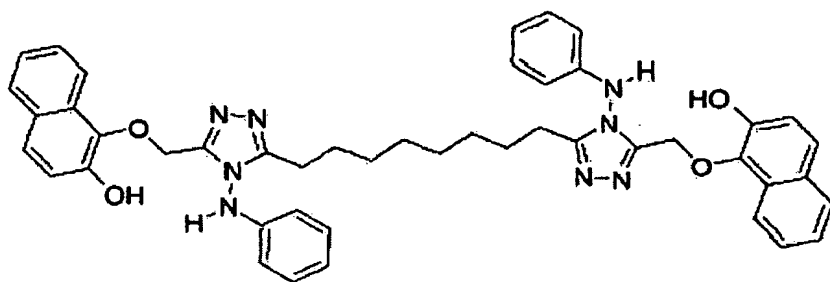
formula VII',



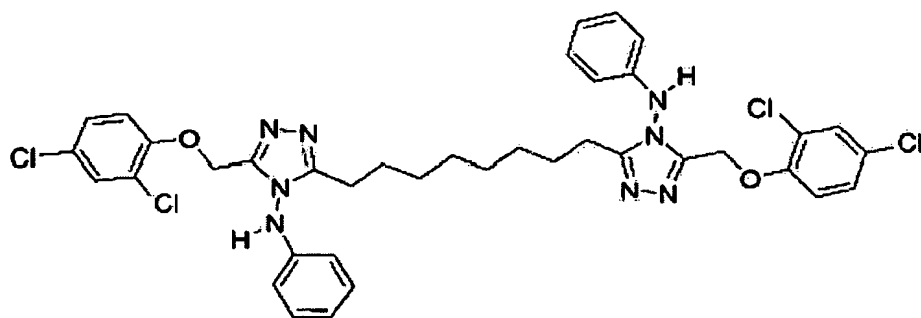
formula VIII',



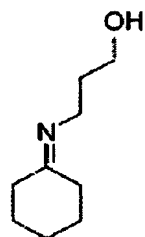
formula IX',



formula X',

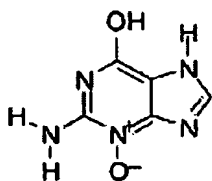


formula XI',



formula XII',

or



formula XIII',

or a pharmaceutically acceptable salt thereof.

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The discussion herein sometimes refers to a compound as having a structure of formula I-VIII, XII or XIII, or formula I' - XIII'. It is to be understood that, unless the context clearly dictates otherwise, a pharmaceutically acceptable salt of the compound is also included.

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In one embodiment, the modulation is the *inhibition* of an AM peptide activity, and the compound is represented by one of formula I through formula VII or, more particularly, the compound is represented by one of formula I' through formula VII'. The activity that is inhibited may be, *e.g.*, stimulation of the level of intracellular cAMP, vasodilation, or the like. Another embodiment is a method for treating a condition that is

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mediated by over-expression and/or -activity of AM, comprising administering to a patient in need of such treatment an effective amount of a compound of formula I, II, III, IV, V, VI, VII, I', II', III', IV', V', VI', or VII'. Among suitable conditions for such treatment are type 2 diabetes or cancer.

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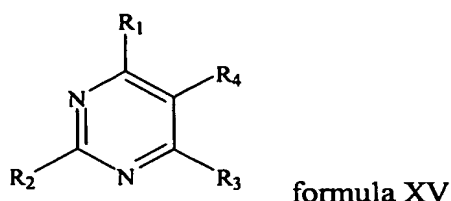
In another embodiment, the modulation is the *stimulation* of an AM peptide activity, and the compound is represented by one of formula VIII, XII or XIII or, more particularly, the compound is represented by one of compound VIII', IX', X', XI', XII' or XIII'. The activity that is inhibited may be, *e.g.*, stimulation of the level of intracellular cAMP, vasodilation, or the like. Another embodiment is a method for treating a condition that is mediated by under-expression and/or -activity of AM, comprising

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administering to a patient in need of such treatment an effective amount of a compound of formula VIII, XII, XIII, VIII', IX', X', XI', XII' or XIII'. Among suitable conditions for such treatment are renal or cardiovascular disease, sepsis, or central nervous system ischemia.

In embodiments of the preceding methods to inhibit or stimulate AM, the peptide and the compound are in an animal, such as a mammal (*e.g.*, following the administration of the compound to the animal *in vivo*), or the peptide and the compound are *in vitro* (not in an animal).

In another embodiment, the invention relates to a method for modulating an activity of a gastrin releasing peptide (GRP) peptide, comprising contacting the peptide with an effective amount of a compound of:  
formula XIV,



wherein R<sub>1</sub> is: 
$$\begin{array}{c} -R_5 - (CH_2)_n - CHOH \\ | \qquad \qquad | \\ H \qquad \qquad R_6 \end{array}$$

wherein R<sub>5</sub> is N, S or O; R<sub>6</sub> is H or CH<sub>3</sub>; and n is 1-4;

R<sub>2</sub> is NH<sub>2</sub>, substituted amino or acetamide;

R<sub>3</sub> is H, halogen, CH<sub>3</sub>, or CF<sub>3</sub>; and

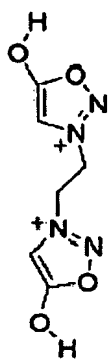
R<sub>4</sub> is H, alkyl, substituted alkyl, alkenyl, alkoxy or halogen,

formula XVI, or

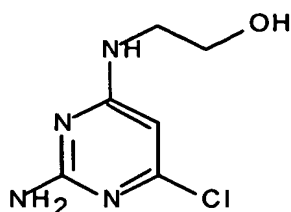
formula XVII,

or a pharmaceutically acceptable salt thereof.

More particularly, a compound of a formula as below is used:

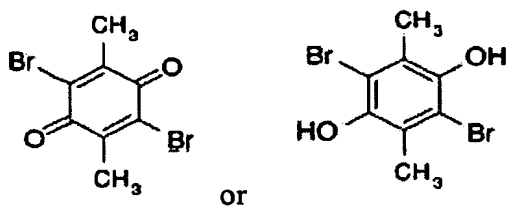


formula XIV',

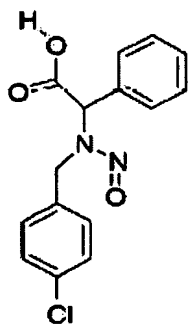


formula XV',

5



formula XVI', or



formula XVII',

10

or a pharmaceutically acceptable salt thereof.

The discussion herein sometimes refers to a compound as having a structure of formula XIV-XVII, or formula XIV' - XVII'. It is to be understood that, unless the context clearly dictates otherwise, a pharmaceutically acceptable salt of the compound is also included.

In one embodiment, the modulation is the *inhibition* of a GRP peptide activity, and the compound is represented by one of formula XIV - XVI or, more particularly, the compound is represented by one of formula XIV' - XVI'. The activity that is inhibited may be, *e.g.*, suppressing food intake, regulating glucose homeostasis, or stimulating hypotension. In another embodiment, the compound is represented by formula XIV, XIV', XVI or XVI', and the activity that is inhibited is, *e.g.*, stimulating intracellular levels of IP<sub>3</sub> or Ca<sup>+2</sup>, or stimulating angiogenesis. Another embodiment is a method for treating a condition that is mediated by over-expression and/or -activity of GRP, comprising administering to a patient in need of such treatment an effective amount of a compound of formula XIV, XV, XVI, XIV', XV', or XVI'. Among suitable treatment methods are treating low blood pressure (hypotension) or an eating disorder (such as anorexia or bulimia), or improving breathing in premature babies (bronchopulmonary dysplasia). In another embodiment, the compound is of formula XIV, XIV', XVI or XVI', and the treatment method is, *e.g.*, reducing tumor growth.

In another embodiment, the modulation is the *stimulation* of a GRP peptide activity, and the compound is represented by formula XVII or, more particularly, by formula XVII'. The activity that is inhibited may be, *e.g.*, stimulating intracellular levels of IP<sub>3</sub> (inositol phosphate) or Ca<sup>+2</sup>, or stimulating angiogenesis, suppressing food intake, regulating glucose homeostasis, or stimulating hypotension. Another embodiment is a method for treating a condition that is mediated by under-expression and/or -activity of GRP, and/or that would benefit from an increased expression or activity of a GRP activity (such as angiogenesis), comprising administering to a patient in need of such treatment an effective amount of a compound of formula XVII or XVII'. Among suitable conditions for such treatment are obesity, diabetes or hypertension. Furthermore, the method may be a method for treating a condition in which stimulation of angiogenesis is

desirable, *e.g.*, coronary or peripheral artery disease, tissue ischemia, organ or tissue transplantation, and acceleration or enhancing of fracture repair or wound healing.

In embodiments of the preceding methods to inhibit or stimulate GRP, the peptide and the compound are in an animal, such as a mammal (*e.g.*, following the administration of the compound to the animal *in vivo*), or the peptide and the compound are *in vitro* (not in an animal).

In another embodiment, the invention relates to a complex, comprising a compound selected from formula I through VIII, XII, or XIII (or, more particularly, formula I' through formula XIII'), in association with (*e.g.*, bound to) an AM peptide, or comprising a compound selected from formula XIV through formula XVII (or, more particularly, formula XIV' through XVII'), in association with (*e.g.*, bound to) a GRP peptide. The complex may be in an animal, such as a mammal (*e.g.*, following the administration of the compound to the animal *in vivo*), or it may be *in vitro* (not in an animal).

In another embodiment, the invention relates to a complex comprising a compound selected from formula I through VIII, XII, or XIII (or, more particularly, formula I' through formula XIII'), in association with (*e.g.*, bound to) a blocking antibody of AM, or comprising a compound selected from formula XIV to formula XVII (or, more particularly, formula XIV' through XVII'), in association with (*e.g.*, bound to) a blocking antibody of GRP. The complex may be in an animal, such as a mammal, or it may be *in vitro* (not in an animal).

In another embodiment, the invention relates to a composition, comprising a compound selected from formula I through VIII, XII, or XIII (or, more particularly, formula I' through formula XIII'), in association with (*e.g.*, bound to) an AM peptide, or comprising a compound selected from formula XIV through formula XVII (or, more particularly, formula XIV' through XVII'), in association with (*e.g.*, bound to) a GRP peptide. The composition may be in an animal, such as a mammal (*e.g.*, following the administration of the compound to the animal *in vivo*), or it may be *in vitro* (not in an animal).

In another embodiment, the invention relates to a composition comprising a compound selected from formula I through VIII, XII, or XIII (or, more particularly,



formula I' through formula XIII'), in association with (*e.g.*, bound to) a blocking antibody of AM, or comprising a compound selected from formula XIV to formula XVII (or, more particularly, formula XIV' through XVII'), in association with (*e.g.*, bound to) a blocking antibody of GRP. The composition may be in an animal, such as a mammal, or it may be  
5 *in vitro* (not in an animal).

In another embodiment, the invention relates to a pharmaceutical composition, comprising a compound selected from formula I through VIII, or formula XII through XVII (or, more particularly, formula I' through XVII') and a pharmaceutically acceptable carrier.

10 In another embodiment, the invention relates to a method (*e.g.*, a diagnostic method) for detecting an AM peptide, comprising contacting a sample suspected of containing the peptide with one or more detectably labeled compounds selected from formula I through VIII, XII, or XIII (or, more particularly, formula I' through formula XIII'), and detecting labeled compound that is associated with (bound to) the peptide; or  
15 for detecting a GRP peptide, comprising contacting a sample suspected of containing the peptide with one or more detectably labeled compounds selected from formula XIV through formula XVII (or, more particularly, formula XIV' through formula XVII'), and detecting labeled compound that is associated with (bound to) the peptide. The detection method may be performed *in vivo* or *in vitro*. Optionally, for example when the detection  
20 is performed *in vivo*, the detectably labeled compound(s) may be in the form of a pharmaceutical composition.

In other embodiments, the invention relates to kits suitable for treating subjects in need of such treatment. In one embodiment, the kit is suitable for treating a subject suffering from a condition mediated by aberrant expression and/or activity of  
25 adrenomedullin (AM); and it comprises one or more of the compounds selected from formula I to VIII, XII, or XIII (or, more particularly, formulas I' through XIII'), or a pharmaceutical composition comprising said compound(s) and a pharmaceutically acceptable carrier and, optionally, a packaging material. In another embodiment, the kit is suitable for treating a subject suffering from a condition mediated by aberrant  
30 expression and/or activity of gastrin releasing peptide (GRP); and it comprises one or more of the compounds selected from formula XIV to formula XVII (or, more

particularly, formula XIV' through XVII'), or a pharmaceutical composition comprising said compound(s) and a pharmaceutically acceptable carrier and, optionally, a packaging material.

In other embodiments, the invention relates to kits suitable for detecting an AM or GRP peptide (*e.g.*, for a diagnostic method). In one embodiment, the kit is suitable for detecting an AM peptide; and it comprises one or more of the compounds selected from formula I to VIII, XII, or XIII (or, more particularly, formula I' through formula XIII'), which is detectably labeled, and, optionally, means to detect the labeled compound associated with (bound to) the peptide. In another embodiment, the kit is suitable for detecting a GRP peptide; and it comprises one or more of the compounds of formula XIV to formula XVII (or, more particularly, formula XIV' through XVII'), which is detectably labeled, and, optionally, means detect the labeled compound associated with (bound to) the peptide. Kits suitable for *in vivo* detection may further comprise a pharmaceutically acceptable carrier.

In another embodiment, the invention relates to a method for inhibiting GRP-mediated angiogenesis in a subject in need of such treatment, comprising administering to the subject an effective amount of an agent that inhibits expression and/or an activity of GRP (*e.g.*, a compound of one of formula XIV - XVI, or a compound of formula XIV' - XVI'), provided that the GRP-mediated angiogenesis is not angiogenesis involved in tumor growth or metastasis; or a method for preventing or treating condition mediated by GRP-mediated angiogenesis in a subject in need of such treatment, comprising administering to the subject an effective amount of an agent that inhibits expression and/or an activity of GRP (*e.g.*, a compound of formula XIV - XVI, or a compound of formula XIV' - XVI'), provided that the condition is not cancer.

Conditions that may be treated with such GRP inhibitors include angiogenesis-mediated conditions (conditions mediated by excessive amounts (pathogenic amounts) of angiogenesis), *e.g.*, arthritis; psoriasis; benign growths caused by rapidly dividing cells; brain ischaemia; vascular diseases; ocular diseases (*e.g.*, diabetic retinopathy); fibrosis; deep venous thrombosis; endometriosis; wrinkles; etc. a more extensive disclosure of suitable conditions is presented elsewhere herein. The term "a GRP inhibitor" or "an AM

inhibitor," as used herein, refers to an agent which inhibits the expression and/or an activity of GRP or AM, respectively.

In another embodiment, the invention relates to a method for inhibiting angiogenesis-mediated tumor growth in a subject in need of such treatment, comprising administering to the subject an effect amount of an agent that inhibits expression and/or an activity of GRP ((*e.g.*, a compound of formula XV, or a compound of formula XV') and detecting or monitoring the reduction in blood vessels (inhibition of angiogenesis).

As noted above, the inventors have developed a two-step screening method to identify agents which modulate an activity of, *e.g.*, a peptide hormone. As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, an agent of the invention that modulates "an" activity of a peptide hormone of interest may modulate one or more such activities.

In the experiments reported herein, modulatory agents were identified for AM and GRP.

As a starting point, molecules of the NCI small molecule (non-peptide) library were screened. This library contains about  $5 \times 10^6$  molecules, which are organized into 2,000 families grouped under the criterion of chemical similarity (Voigt *et al.* (2001) *J. Chem. Inf. Comput. Sci.* 41, 702-712). Structures of the compounds are available at the web site [cactus.nci.nih.gov/ncidb2](http://cactus.nci.nih.gov/ncidb2). Any library of small organic or inorganic molecules, such as molecules obtained from combinatorial and natural product libraries, can be tested and identified by the methods of the invention.

Furthermore, other types of molecules can also be screened, including (1) peptides, such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries, such as is described in Lam *et al.* (1991) *Nature* 354, 82-84; Houghten *et al.* (1991) *Nature* 354, 84-86), and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, and specific derivatives of peptides of interest; (2) antibodies (*e.g.*, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, F(ab')<sub>2</sub>, Fab expression library fragments, and epitope-binding fragments of antibodies); and (3)

phosphopeptides, such as members of random and partially degenerate, directed phosphopeptide libraries, *e.g.*, as in Songyang *et al.* (1993) *Cell* 72, 767-778.

The method of the invention was designed based on the assumption that a neutralizing antibody, such as a monoclonal antibody, binds to an epitope on the peptide that is important, if not critical, for receptor recognition. Without wishing to be bound by any particular mechanism, the inventors appear to have confirmed this assumption by the identification of biologically active compounds capable of modulating the physiology of AM and GRP. In addition, the antibody-based colorimetric screening procedure allows for high throughput formats able to analyze thousands of compounds (or more) in very short periods of time.

The first step in the procedure exemplified herein was to identify compounds that interfered with binding between the peptide and its blocking monoclonal antibody. Some details of this first step in the assay are presented in Example 3. See also the results shown in Fig. 1A. In many cases, active compounds could be identified by the naked eye, even before colorimetric quantification (see Fig. 1B). 2,000 parental compounds of the library were screened using this methodology for AM, and 121 of them caused a significant inhibition in color intensity in a statistically significant fashion.

The inventors also screened the same compounds with a blocking monoclonal antibody against GRP (Chaudhry *et al.* (1999) *Clin. Cancer Res.* 5, 3385-3393), a peptide similar to AM in size and in chemical characteristics. This allowed the evaluation of the specificity of this methodology, as well as the identification of modulatory agents for GRP. Screening the same clinical library, 109 compounds were identified that inhibited color formation to a significant degree. Only 5 of them were also present among the molecules able to interfere with AM, indicating that, in fact, different combinations of peptide-antibody complexes pulled out distinct sets of small molecules. This clearly shows that this methodology is able to discriminate between target molecules.

As is discussed below, many of the compounds identified in this first step of the assay were not useful for modulating receptor-mediated responses. In the experiments reported herein, only 19.8% of the compounds tested for AM, and 4.6% of the compounds tested for GRP, fulfilled this criterion. Nevertheless, this first step allowed for a rapid primary screening of a large number of compounds, and reduced considerably

the number of compounds that must be tested with the more expensive and time-consuming cell-based screen.

Since the first step of the screening strategy was based on the ability of the test molecules to interfere with the binding between the peptide and its antibody, it was possible that not all these molecules would also modify the binding between the peptide and its receptor, even though the monoclonal antibodies used were shown to be neutralizing. To investigate functional consequences of the molecules identified in the first step of the screen, all the “positive” compounds were subjected to an analysis of their ability to modify the production of the intracellular second messenger elicited by the specific receptor system.

All the compounds chosen with the primary AM screening were analyzed with a cAMP assay. Details of this second step of the assay are present in Example 4a. From the initial 121 compounds, 24 were able to significantly modulate the amount of cAMP induced by 100 nM AM in Rat2 cells, whereas the other 97 did not modify the cAMP response to AM. Interestingly, some of these compounds reduced the cAMP levels (acted as antagonists) whereas others actually elevated intracellular cAMP levels over the levels induced by AM alone, identifying them as superagonists (Fig. 2A, Table 1). In the absence of AM, none of the compounds elicited any response (Fig. 2A), suggesting that the mechanism of action includes binding of the small molecule to AM rather than to the receptor. That is, the molecules acted as superagonists rather than as agonists. These responses were dose-dependent, with drug responses seen with chemical concentrations as low as 10 nM (Fig. 2B).

Peptide	Action on second messengers	Code <sup>I</sup>	Biological activity
AM	Antagonists	16311 (compound I)	Elevates blood pressure
		37133 (compound II)	
		48747 (compound III)	Elevates blood pressure
		89435 (compound IV)	Elevates blood pressure

		28086 (compound V')	
		79422 (compound VI')	
		50161 (compound VII')	
	Superagonists	697165 (compound VIII')	
		697162 (compound IX')	
		697168 (compound X')	
		697169 (compound XI')	
		128911 (compound XII')	Reduces blood pressure
		145425 (compound XIII')	Reduces blood pressure
GRP	Antagonists	54671 (compound XIV')	
		77427 (compound XV')	Inhibits cord formation
		112200 (compound XVI')	
	Superagonist	372874 (compound XVII')	

Table 1.

Table 1 shows compounds that induced consistent effects on modulating second messenger activation by AM or GRP. Some of them were tested for biological activity (4<sup>th</sup> column). <sup>1</sup> The chemical structures of these compounds can be found at the web site: [cactus.nci.gov/ncidb2](http://cactus.nci.gov/ncidb2).

5

The primary difference between AM and CGRP receptors is the nature of the particular RAMP that is associated to CRLR. When the active compounds for AM were added to a CGRP receptor-containing cell in the presence of synthetic CGRP, no effect was observed (Fig. 2D), demonstrating the specificity of these compounds for the binding between AM and its receptor.

For the compounds that showed promising behavior by both screening steps, close structurally related chemical family members were also evaluated. The prediction was that a similar chemical structure would exhibit similar biological behavior. To test this, the following related family members were tested: original compound VIII' and related compounds IX', X' and XI'. In most cases, this analysis produced compounds with stronger activity than the original substance (Fig. 2C), suggesting that grouping compounds based on their chemical similarity could be useful to predict their potential biological activity. That is, one could use a modular approach to the screening process, beginning with the leading 2,000 compounds and then with other members of the promising families. This strategy, combined with the high throughput antibody-based primary screening, allowed for a complete preliminary search of the whole library in a matter of days.

In a similar approach, the small molecules that were identified in the first screening step with the GRP antibody were characterized by their ability to modify IP<sub>3</sub> or Ca<sup>2+</sup> levels induced by synthetic GRP in cells containing its receptor (see Example 4b and Fig. 3). Again, both antagonist and superagonist molecules were identified. As was the case with modulators of AM, the GRP-interfering small molecules by themselves did not produce any change in IP<sub>3</sub> levels (Fig. 3A). In the Ca<sup>2+</sup> assay, 1 nM GRP produced a marked elevation of intracellular Ca<sup>2+</sup> in H1299 cells (Fig. 3B), but pre-exposure of the cells to the identified antagonists greatly reduced the Ca<sup>2+</sup> spike amplitude (Fig. 3C). The compounds that showed a consistent behavior with either the AM or the GRP systems are summarized in Table 1.

To validate the biological activity of some of the small molecules selected above, several assays were performed. For example, an important function of AM is the regulation of blood pressure. As is described in more detail in Example 5, injection of screen-selected AM superagonists (at 20 nmols/Kg) in hypertensive rats induced a

profound and long-lasting decrease from basal levels in blood pressure ranging from 50 to 70 mm Hg (Fig. 4A,B). Vehicle alone (DMSO in PBS) at the same concentration did not alter blood pressure (Fig. 4A). On the other hand, when screen-selected small molecule AM antagonists were injected into normotensive animals, also at 20 nmols/Kg, an elevation in blood pressure was observed (Fig. 4C). The blood pressure profile generated by the superagonists was similar to the one elicited by the peptide itself (that was used as a control in Fig. 4B), suggesting that these small molecules may be enhancing the effect of circulating AM. Similar *in vivo* effects are expected for the remaining modulators of AM. Further studies to elucidate the mechanism of action of AM modulators are presented in Example 11.

Biological activity of small molecule GRP antagonists is also demonstrated in the Examples. The influence of some of the GRP antagonists was analyzed in angiogenic models. Example 6 demonstrates that GRP can induce cord formation *in vitro*, in a culture of endothelial cells grown on Matrigel, and that a small molecule GRP inhibitor of the invention greatly reduces the complexity of the tubular lattice. Example 7 demonstrates, in an *in vivo* model of directed angiogenesis (DIVAA), that GRP exhibits angiogenesis potential *in vivo*, and that this angiogenesis is inhibited by a small molecule GRP antagonist of the invention. Growth inhibition assays show that a small molecule GRP inhibitor inhibits the growth of a lung cancer cell line *in vitro* (Example 8), and that it reduces the number of colonies in a clonogenic assay (Example 9). The inhibitors also display inhibitory activity in an *in vivo* assay of tumor growth in mice (Example 10). The experiments in these examples were carried out primarily with the small molecule GRP inhibitor, compound XV' (77427). Similar effects are expected for the remaining antagonists of GRP.

In a preliminary analysis, the inventors have identified some elements that appear to be conserved among some of the modulatory agents identified herein. Without wishing to be bound by any particular model, it is suggested that the modulatory agents fall into several "families" of structures. A careful analysis of the chemical structures of some of the active compounds for AM reveals some common characteristics. The most active antagonists (*e.g.*, compounds of formula I' (16311), formula IV' (89435) and formula VII' (50161)) have in common an aromatic ring separated from a three-



substituted nitrogen by 4 elements. There is also a hydroxy group at 2 or 3 elements from the nitrogen. Several compounds with superagonist activity (*e.g.*, compounds of formula XIII' (145425), formula XII' (128911) and formula VIII' (697165)) share the presence of nitrogenated heterocycles with oxygen atoms at similar distances. Nevertheless, the surprising simplicity of compound XII' (128911) suggests that the activity may be due just to the presence of a nitrogen with  $sp^2$  hybridization situated at a determined distance from the oxygen.

The resolution of the three-dimensional structures of the AM-AM receptor complex and the GRP-GRP receptor complexes should allow one to identify more precisely the binding sites of the small molecules identified herein and to introduce direct design modifications of these molecules to fit the active site more closely. Such methods are conventional. See, *e.g.*, rational design methods in Ghosh *et al.* (2001) *Curr. Cancer Drug Targets* 1, 129-140. Additional optimization can be obtained by generating additional compounds by combinatorial chemistry, for example by modifying slightly the chemical backbone identified here with different radicals. Such methods are conventional. See, *e.g.*, Gray *et al.* (1998) *Science* 281, 533-538 and Poyner *et al.* (2002) *Pharmacol Rev* 54, 233-246.

The invention relates to a method to identify an agent that modulates (*e.g.*, modulates an activity of) a peptide which interacts specifically with a receptor, such as a peptide hormone, preferably AM or GRP, comprising

- a) contacting the peptide, a blocking antibody of the peptide, and a putative binding-inhibitory agent,
- b) detecting binding of the peptide to the antibody, and
- c) selecting an agent which inhibits (*e.g.*, disrupts) said binding, compared to the binding in the absence of the putative binding-inhibitory agent, thereby identifying a binding-inhibitory agent.

The binding-inhibitory agent may be an antagonist or an agonist of the peptide. Preferably, the putative binding-inhibitory agent is a non-peptide small molecule. In one embodiment, the method is a high throughput method (assay).

In a preferred embodiment, the above method further comprises

d) contacting a binding-inhibitory agent identified as above, the peptide, and a cell that comprises a receptor for the peptide,

e) detecting the amount in the cell of a second messenger induced by the peptide, and

5 f) selecting an agent that modulates the amount of the second messenger in the cell, compared to the amount in the cell in the absence of the agent, thereby identifying a modulatory agent.

10 The modulatory agent may be an antagonist or an agonist (*e.g.*, a superagonist) of the peptide. For example, the agent may be an agonist or antagonist of an activity of the peptide, such as its binding to a receptor, the stimulation (expression) of a second messenger, or any of the other activities described elsewhere herein. In one embodiment, the cell comprises a receptor for AM, and the second messenger is AM-induced cAMP. In another embodiment, the cell comprises a receptor for gastrin releasing hormone (GRP), and the second messenger is GRP-induced IP<sub>3</sub> or Ca<sup>++</sup>. Preferably, the putative  
15 binding-inhibitory agent is a non-peptide small molecule. In one embodiment, the method is a high throughput method (assay).

Any of the preceding methods for identifying putative modulatory agents may further comprise additional steps, some of which are discussed elsewhere herein. The invention also relates to modulatory agents which are identified and/or characterized by a  
20 method of the invention, particularly small, non-peptide, molecules that are encompassed by one of the generic structures identified herein.

Modulatory agents of the invention (*e.g.*, small molecule non-peptide compounds) can be prepared (*e.g.*, synthesized) fully conventionally, using known reaction chemistry, starting from known materials or materials conventionally preparable. Procedures for  
25 synthesizing small molecule, non-peptide, compounds can readily produce gram amounts of a compound of interest. Many compounds of the invention are readily available from standard sources, such as chemical supply houses, or can be generated from commercially available compounds by routine modifications.

30 The present invention also relates to useful forms of the compounds as disclosed herein, such as pharmaceutically acceptable salts and prodrugs of all the compounds of the present invention. Pharmaceutically acceptable salts include those obtained by

reacting the main compound, functioning as a base, with an inorganic or organic acid to form a salt, for example, salts of hydrochloric acid, sulfuric acid, phosphoric acid, methane sulfuric acid, camphor sulfonic acid, oxalic acid, maleic acid, succinic acid and citric acid. Pharmaceutically acceptable salts also include those in which the main compound functions as an acid and is reacted with an appropriate base to form, *e.g.*, sodium, potassium, calcium, magnesium, ammonium, and chlorine salts. Those skilled in the art will further recognize that acid addition salts of the claimed compounds may be prepared by reaction of the compounds with the appropriate inorganic or organic acid via any of a number of known methods. Alternatively, alkali and alkaline earth metal salts are prepared by reacting the compounds of the invention with the appropriate base via a variety of known methods..

The following are further examples of acid salts that can be obtained by reaction with inorganic or organic acids: acetates, adipates, alginates, citrates, aspartates, benzoates, benzenesulfonates, bisulfates, butyrates, camphorates, digluconates, cyclopentanepropionates, dodecylsulfates, ethanesulfonates, glucoheptanoates, glycerophosphates, hemisulfates, heptanoates, hexanoates, fumarates, hydrobromides, hydroiodides, 2-hydroxy-ethanesulfonates, lactates, maleates, methanesulfonates, nicotines, 2-naphthalenesulfonates, oxalates, palmoates, pectinates, persulfates, 3-phenylpropionates, picrates, pivalates, propionates, succinates, tartrates, thiocyanates, tosylates, mesylates and undecanoates.

Preferably, the salts formed are pharmaceutically acceptable for administration to mammals. However, pharmaceutically unacceptable salts of the compounds are suitable as intermediates, for example, for isolating the compound as a salt and then converting the salt back to the free base compound by treatment with an alkaline reagent. The free base can then, if desired, be converted to a pharmaceutically acceptable acid addition salt.

Agents of the invention may be used in therapeutic methods for conditions (including pathogenic conditions, or diseases) that are mediated by aberrant expression and/or activity of a peptide hormone, such as AM or GRP, and/or for conditions (including non-pathogenic conditions) that respond to an increase or decrease in the expression or activity of the peptide hormone. The term "aberrant" expression and/or

activity, as used herein, includes expression or activity that is higher or lower than a base line value, such as the amount present in a subject who does not exhibit symptoms of the condition, or who does not exhibit a predisposition to the condition. The expression or activity may be an "under" -expression or -activity, or an "over" -expression or -activity.

5 When aberrant expression results in undesirable symptoms, the condition is sometimes said to be a pathological condition.

The therapeutic methods include diagnosis, treatment, prevention, and/or amelioration of symptoms of any of a variety of conditions (*e.g.*, pathological conditions) in a subject, or modulation of physiological conditions (*e.g.*, non-pathological conditions, 10 such as the stimulation or inhibition of appetite), which are associated with AM or GRP activity. The subject (*e.g.*, a patient) can be any suitable animal, including mammals, birds, reptiles, fish, amphibians, etc. Suitable subjects include, *e.g.*, experimental animals (such as mice, rats, guinea pigs, rabbits, fish, frogs, etc.), pets, farm animals (such as cows, pigs, horses, birds such as chickens or geese, etc.), and primates, especially 15 humans.

With regard to agents that modulate AM activity, AM levels are dysregulated in many pathologies (*e.g.*, in humans), such as hypertension, heart failure, sepsis, cancer, or diabetes, when compared to healthy controls. See, *e.g.*, the AM-mediated conditions discussed in U.S. patent application 20020055615. This correlation, together with 20 experimental actions of AM in relevant model systems, implicates this molecule in the pathophysiology of such conditions. Interestingly, changes in AM levels may have apparently paradoxical effects on a patient's health, depending on the particular disease studied.

For example, elevated AM expression seems to exert a protective role in renal and 25 cardiovascular diseases, sepsis, and in central nervous system ischemia. Without wishing to be bound by any particular mechanism, it is suggested that overexpression of AM is protective due to its vasodilator activity. An agent that acts as an agonist or superagonist of AM can be used to treat or prevent conditions that are ameliorated by the expression of AM, such as, *e.g.*, vascular diseases, trauma, malignant hypotension, catecholamine 30 disorders, or the other conditions noted above.

In other circumstances, elevated AM expression appears to worsen a pathological condition, such as the progression of type 2 diabetes and cancer. In diabetic rats, injection of AM results in a reduction of circulating insulin levels and a concomitant hyperglycemia, whereas application of a monoclonal antibody against AM lowers glucose levels and ameliorates postprandial hyperglycemia. AM antagonists of the invention may be used to treat diabetes, *e.g.*, by regulating insulin secretion and/or blood glucose metabolism. In cancer cells, AM acts as a tumor survival factor. This tumor survival may be influenced by various activities of AM, such as elevation of tumor cell growth, circumventing apoptosis, increasing migration, and enhancement of angiogenesis. Among the types of neoplastic transformation (*e.g.*, cancerous cells) that can be treated by AM antagonists of the invention are, *e.g.*, adrenal, nervous system (*e.g.*, brain tumors, such as gliomas, astrocytomas or neuroblastomas), renal, lung (*e.g.*, small cell lung cancer), pancreatic, gastric, gastrointestinal, lung (*e.g.*, small cell lung cancer), colon, colorectal, prostate, ovarian and breast cancerous cells, and chondrosarcoma, as well as other types of neoplastic diseases discussed herein with reference to GRP antagonists. An agent that acts as an antagonist of AM can be used to treat or prevent conditions that are rendered worse by the expression of AM, such as the conditions noted above, or others.

Additional conditions that can be diagnosed, treated, and/or prevented with antagonists or agonists (*e.g.*, superagonists) of AM will be evident to the skilled worker. Among the physiological effects of AM are bronchodilation, regulation of hormone secretion, neurotransmission, antimicrobial activities, and regulation of cell growth and migration. One of skill in the art will recognize a variety of conditions that are mediated by these, or other, effects. Among the treatment methods for which agents of the invention are suitable are, *e.g.*, treating conditions related to pregnancy (*e.g.*, diagnosing and/or treating preeclampsia or promoting fetal growth); regulating activity in areas of the central nervous system (*e.g.*, regulation of neurotransmission or neuron growth, such as in, *e.g.*, Alzheimer's disease); lessening or inhibiting the allergic response due to the degranulation of mast cells; treating bacterial and fungal infections by inhibiting or preventing bacterial or fungal growth; facilitating the healing of chafed skin, skin lesions, wound repair, and surgical incisions (*e.g.*, by applying to the surface of the skin of a

subject an amount of one or more of the agents of the present invention effective to facilitate healing); and promoting organ and bone development.

For a further discussion of some conditions that can be diagnosed, treated and/or prevented with AM antagonists or agonists, and suitable methods that can be applied to use of the modulatory compounds of the invention, see U.S. patent application 20020055615 (Cuttitta *et al.*).

With regard to agents that modulate GRP activity, GRP levels are dysregulated in many pathologies (*e.g.*, in humans), such as cancers, compared to healthy controls. The inventors have used a neutralizing monoclonal antibody against GRP in phase I/II clinical trials of previously treated small cell lung cancer patients (Chaudhry *et al.* (1999) *Clin. Cancer Res.* 5, 3385-3393). The results of that trial, including a curative complete response, suggest that inhibitors of GRP biology may be very useful in addressing clinical problems.

Several endocrine peptides have been shown to promote angiogenesis. Here, the inventors demonstrate that GRP is another endocrine peptide which promotes angiogenesis (is a pro-angiogenic factor). Angiogenesis is a complex process that requires endothelial cell growth and migration, extracellular matrix remodeling, formation of tubular structures, and loop formation, among other mechanisms. The studies reported in Example 6 show the ability of an exemplary small molecule to interfere with the cord formation ability of GRP. GRP by itself promoted the development of a complex meshwork made of pseudo-capillaries. The simultaneous application of compound XV' (77427) resulted in a marked decrease in the complexity of the tubular network, indicating a utility of this compound in antiangiogenic interventions. Example 7 shows by an *in vivo* assay that GRP exhibits angiogenic potential *in vivo*, and that this angiogenesis can be inhibited by compound XV'. That is, GRP is a potent angiogenic factor, which acts directly to stimulate angiogenesis (rather than through intermediate effects, such as the stimulation of angiogenic factors such as VEGF or bFGF). Example 10 shows that an exemplary GRP antagonist of the invention (compound XV') effectively inhibits tumor-induced angiogenesis in an *in vivo* assay. In the experiments described in this example, a human tumor cell line is transplanted into a

mouse (as a xenograft), and the small molecule antagonist effectively blocks tumor growth.

The demonstrations herein that GRP is a pro-angiogenic factor, and that at least two types of GRP inhibitors - small (non-peptide) molecules and monoclonal antibodies - can inhibit angiogenesis, suggests that any of a broad genus of types of GRP inhibitors can be used to inhibit angiogenesis. Inhibitors of expression of GRP (*e.g.*, antisense molecules, siRNAs, etc) or inhibitors of GRP expression (*e.g.*, antibodies, such as monoclonal antibodies specific for GRP, or small molecule, non-peptide, antagonists of GRP) can be used. Preferably, the small molecule inhibitor is a compound of formula XV or XV'.

Among the many types of angiogenesis-mediated conditions (conditions mediated by aberrant angiogenesis) which can be treated with GRP inhibitors are, *e.g.*, arthritis (*e.g.*, rheumatoid arthritis); psoriasis; benign growths caused by rapidly dividing cells (*e.g.*, noncancerous melanomas); brain ischaemia; vascular diseases (*e.g.* atherosclerosis, myocardial angiogenesis, post-balloon angioplasty, vascular restenosis, neointima formation following vascular trauma, vascular graft restenosis, coronary collateral formation, deep venous thrombosis, ischemic limb angiogenesis); ocular diseases involving ocular neovascularization or related ocular diseases and disorders (*e.g.*, diabetic neovascularization, neovascular glaucoma, macular degeneration, diabetic and other retinopathy, retrolental fibroplasia and corneal diseases); fibrosis (*e.g.*, fibrosis associated with a chronic inflammatory condition, lung fibrosis, chemotherapy-induced fibrosis, wound healing (*e.g.*, of chronic wounds) with scarring and fibrosis; deep venous thrombosis; endometriosis; wrinkles (*e.g.*, UVB-induced wrinkles), etc. In general, anti-angiogenic agents of the invention may be used to treat any disease or condition in which angiogenesis or cell migration/invasiveness is pathogenic. In some embodiments, the angiogenesis-mediated condition is not tumor growth.

The studies shown in Examples 6 through 10 are performed with an exemplary GRP antagonist of the invention (a compound of formula XV'). The other GRP antagonists of the invention are also expected to exhibit similar effects.

One embodiment of the invention is a method for inhibiting angiogenesis-mediated tumor growth in a subject in need of such treatment, comprising administering

to the subject an effective amount of an agent that inhibits the expression and/or an activity of GRP (*e.g.* a compound of the invention). In some embodiments, *e.g.*, when the GRP inhibitor is a compound of formula XV or XV', an additional step is added which reflects the ability of GRP inhibitors to inhibit angiogenesis (*e.g.*, detecting or  
5 monitoring the reduction in blood vessels (inhibition of angiogenesis)).

A variety of types of neoplastic diseases (*i.e.*, cellular proliferative diseases) can be treated with GRP antagonists (*e.g.*, tumor growth can be reduced). Among the proliferative conditions that benefit from administration of the agents of the invention are, *e.g.*, sarcomas, carcinomas, lymphomas, malignant melanomas, and benign growths  
10 caused by rapidly dividing cells. The disease or condition being treated may be primary tumor growth, tumor invasion or metastasis. Cancers of the types discussed above with regard to AM antagonists, *e.g.*, adrenal, nervous system (*e.g.*, brain tumors, such as gliomas, astrocytomas or neuroblastomas), renal, lung, pancreatic, gastric, gastrointestinal, lung, colon, colorectal, prostate, ovarian and breast cancerous cells, and  
15 chondrosarcoma, are included.

Conditions which are mediated by an under-expression of a GRP-mediated condition can also be treated with agents of the invention. That is, GRP agonists (*e.g.*, superagonists) are useful for treating conditions in which *increased* angiogenesis is desirable. Such conditions include, *e.g.*, coronary or peripheral artery disease; any form  
20 of tissue ischemia resulting from vascular occlusion, vascular disease or surgery (*e.g.*, peripheral limb ischemia or hepatic arterial occlusion in liver transplantation); organ or tissue transplantation (*e.g.*, liver organogenesis, or in conjunction with cellular therapy and transplantation of pancreatic islet cells in the treatment of diabetes, as vascular endothelium acts to stimulate or induce pancreatic organogenesis and insulin production  
25 by pancreatic beta cells); and acceleration or enhancing of fracture repair or wound healing (including recovery from surgical wounds, and treatment of chronic wounds with scarring and fibrosis).

GRP is involved in a number of other physiological functions, which will be evident to a skilled worker. These functions include, *e.g.*, the suppression of food intake, regulation of glucose homeostasis, regulation of short-term memory, and enhancement of  
30 hypotension. Among the conditions that can be treated or prevented with GRP



antagonists are, *e.g.*, eating disorders (such as anorexia or bulimia, in which stimulation of food intake is desirable) and low blood pressure (hypotension). GRP antagonists can also be used commercially when it is desirable to increase the weight of animals designated for meat production, such as cows or pigs. GRP antagonists can also be used to improve breathing in premature babies (bronchopulmonary dysplasia), or to treat gastrointestinal disorders, such as peptic ulcer and pancreatitis. Among the conditions which can be treated or prevented with GRP agonists (*e.g.*, superagonists) are, *e.g.*, obesity, diabetes or hypertension. Other conditions suitable for treatment with GRP antagonists or agonists will be evident to the skilled worker. For a discussion of some physiological functions of GRP, and some disease conditions that can be treated or prevented with antagonists or agonists of GRP, see, *e.g.*, Mantey *et al.* (2001) *The Journal of Biological Chemistry* 276, 9219-9229; Merali *et al.* (1999) *Neuropeptides* 33, 376-386; and Ohki-Hamazaki *et al.* (1997) *Nature* 390, 165-169.

Any of the suggested treatment or prevention methods using AM or GRP antagonists or agonists (*e.g.*, superagonists) may be combined with other therapeutic modalities, and combinations of the agents of the invention may be used.

In some of the inventive methods for modulating an activity of a peptide, or for detecting a peptide, the peptide is "contacted" with a modulatory agent of the invention. This contacting may be achieved in a subject (*in vivo*) or outside of an animal (*in vitro*). Suitable methods for contacting are conventional and well-known in the art. For example, a peptide can be contacted with a compound in a cell (either *in vivo* or *in vitro*) by introducing the compound by injection, such as microinjection, electroporation, sonoporation, a gene gun, liposome delivery (*e.g.*, Lipofectin<sup>®</sup>, Lipofectamine<sup>®</sup> (GIBCO-BRL, Inc., Gaithersburg, MD), Superfect<sup>®</sup> (Qiagen, Inc. Hilden, Germany) and Transfectam<sup>®</sup> (Promega Biotec, Inc., Madison, WI), or other liposomes developed according to procedures standard in the art), or receptor-mediated uptake and other endocytosis mechanisms.

In methods of treatment according to the invention, an effective amount of an agent of the invention is administered to a subject. The term "an effective amount," as used herein, means an amount that elicits a detectable response (*e.g.*, amelioration of a symptom or a physiological response); the degree of the response can be minimal,

provided that it is detectable. Similarly, in methods for modulating an activity of a peptide, an effective amount of an agent of the invention is contacted with the peptide. An "effective amount" in this context means an amount that elicits a detectably amount of modulation.

5 In methods of treatment, the agent can be administered (delivered) by any of a variety of conventional procedures. Suitable routes of administration include parenteral and non-parenteral routes. Parenteral routes include, *e.g.*, intravenous, intraarterial, intraportal, intramuscular, subcutaneous, intraperitoneal; intraspinal, intrathecal, intracerebroventricular, intracranial, intrapleural or other routes of injection. Non-  
10 parenteral routes include, *e.g.*, oral, nasal, transdermal, pulmonary, rectal, buccal, vaginal, ocular. Topical administration is desirable, for example, when the condition to be treated is presented on an accessible surface, such as a mucosal surface. Topical administration to the skin (cutaneous delivery) is particularly useful for the treatment of, *e.g.*, psoriasis or skin cancer. In a preferred embodiment, the administration is timed,  
15 slow-release, aerosolized administration. Administration may also be by continuous infusion, local administration, "directed systemic" administration, sustained release from implants (gels, membranes or the like), and/or intravenous injection.

Dosages to be administered can be determined by conventional procedures known to those of skill in the art. See, *e.g.*, *The Pharmacological Basis of Therapeutics*,  
20 Goodman and Gilman, eds., Macmillan Publishing Co., New York. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Factors to be considered include the activity of the specific therapeutic agent involved, its metabolic stability and length of action, mode and time of administration, drug combination, rate of excretion, the species being treated, and  
25 the age, body weight, general health, sex, diet, and severity of the particular disease-states of the host undergoing therapy. Dosages can be selected in a manner customary for treatment with comparable agents for the same condition.

The agents of the invention may be formulated as pharmaceutical compositions, with any of a variety of conventional, pharmaceutically acceptable carriers, diluents  
30 and/or excipients. For suitable components and methods of preparing pharmaceutical compositions, see, *e.g.*, *Remington's Pharmaceutical Sciences*, 18th ed., Mack Publishing

Company (1990); the Handbook of Pharmaceutical Excipients, American Pharmaceutical Association (current edition); and Pharmaceutical Dosage Forms: Tablets (Lieberman, Lachman and Schwartz, eds., current edition, published by Marcel Dekker, Inc.)

5 Agents of the invention may also be used in detection (*e.g.*, diagnostic) procedures. For example, compounds of the invention can be labeled with conventional labels, using conventional procedures, and then used to detect AM or GRP, *in vivo* or *in vitro* (*ex vivo*). Compounds in which a detectable label is present are sometimes referred to herein as "detectably labeled" compounds. Methods (means) of labeling the  
10 compounds and detecting the detectable labels (*e.g.*, detecting labeled compounds that have become associated with (*e.g.*, bound to) the peptide) are conventional and well-established. The detection may be direct, or indirect (*e.g.*, as in some enzymatic detection methods). In some embodiments, the detection is quantitative.

With regard to *in vivo* imaging, since both AM and GRP are turned over rapidly  
15 in the body, there is little circulating AM or GRP. Thus, *in vivo* detection (imaging) to determine where AM or GRP is localized in an organism can indicate the site at which the peptide is produced. Labeled compounds of the invention can be used in any situation in which a tracer of AM or GRP is desirable. Suitable labels will be evident to a skilled worker and include, *e.g.*, heavy metals, which can be detected in PET scans, and  
20 radioactive labels, such as <sup>131</sup>I or other short-lived radioactive tracers. Because many cancers are associated with the production of large amounts of AM or GRP, detection (diagnostic) methods as above with AM or GRP modulatory compounds are useful for detecting the presence and/or location of a cancer. Other uses of such *in vivo* detection (diagnostic) methods will be evident to the skilled worker.

25 As for *in vitro* methods (assays), a compound of the invention can be labeled with a conventional detectable label, such as a fluor or an enzyme (*e.g.*, lactoperoxidase, alkaline phosphatase, or beta galactosidase), and then contacted with a tissue sample (such as a pathology sample) in order to visualize the presence of the peptide (*e.g.*, to identify a cancerous tissue). The compounds of the invention can be used in a variety of  
30 *in vitro* assay procedures, not only to detect the presence of a peptide of interest, but also to quantitate the amount of the peptide. For example, the compound can substitute for a

monoclonal antibody in a conventional radioimmunoassay. In addition, the modulatory agents can be used for pharmacological drug design. For example, by analyzing the three dimensional structure of a complex between an AM or GRP peptide, or a blocking antibody for the peptide, and a compound identified herein or a variant thereof, using

5 NMR or NMR imaging, one can screen and/or characterize variants that are more effective antagonists or agonists than the starting compound. (The compounds identified herein can serve as comparative controls in such a method.) Other suitable *in vitro* methods in which compounds of the invention can be used will be evident to the skilled worker.

10 Detection methods of the invention can be used to detect (*e.g.*, diagnose or monitor) any of the conditions described elsewhere herein, or others, which are mediated by aberrant expression and/or activity of AM or GRP. For example, one can monitor a condition (*e.g.*, a disease condition) by measuring the amount of AM or GRP in a sample, wherein the presence of the AM or GRP indicates the existence of, or predisposition to,

15 the condition. Examples of conditions that can be diagnosed or monitored by methods of the invention include, but are not limited to, diabetes; renal diseases, such as severe uremia; bone diseases, such as neoplastic disease; skin diseases; and blood related diseases, such as leukemia.

20 In view of the tight association of the small molecules of the invention to AM or GRP, or, in some cases, to the receptors for AM or GRP, the small molecules of the invention can also be used to target additional therapeutic agents to cells in need of such treatment (cells which express AM or GRP, or receptors for those peptides). Suitable therapeutic agents (*e.g.*, toxins such as ricin, diphtheria toxin, etc., to target tumor cells)

25 will be evident to the skilled worker. For some examples of suitable therapeutic agents, see, *e.g.*, US application 20020176819 and WO00/54805.

In another aspect of the invention, modulatory compounds of the invention are found in complexes with (or are in compositions with) the AM or GRP peptides, or with

30 blocking antibodies specific for those peptides. In the complexes (or compositions) of the invention, the compounds associate with (*e.g.*, bind to) the peptides or antibodies by

any of a variety of means that are well-known to skilled workers. The types of association include, *e.g.*, covalent bonds or non-covalent bonds (*e.g.*, passively adsorbed, such as by electrostatic forces, ionic or hydrogen bonds, hydrophilic or hydrophobic interactions, Van der Waals forces, etc.).

5           Complexes of the invention can provide tools for the characterization of receptors, binding proteins, and other binding sites, and can help elucidate the mechanism of action of the peptide hormones. For example, because the blocking antibodies described herein mimic the receptors to which the peptide hormones bind, the antibodies can serve as surrogate receptors. Thus, small molecule/antibody complexes can serve as artificial  
10   ligand/receptor complexes. See also the types of studies described in Poyner *et al.* (2002) *Pharmacol. Rev.* 54, 233-246 and Pio *et al.* (2002) *Microsc. Res. Tech.* 57, 23-27. In another embodiment, a complex between a peptide or antibody and a compound of the invention can be used to identify and/or characterize other compounds that exhibit more effective antagonist of agonist activity, *e.g.*, as discussed above.

15           When a modulatory compound of the invention is administered to an animal, a complex of the compound and AM or GRP may form *in vivo* (in the animal).

          An *in vitro* complex of a modulatory compound of the invention and a peptide or blocking antibody of the invention is sometimes referred to herein as an "isolated" complex. As used herein, the term "isolated," when referring, *e.g.*, to a complex of the  
20   invention, means that the material is not in its naturally occurring form, is generated artificially *in vitro*, and/or is isolated or separated from at least one other component with which it is naturally associated. For example, a naturally-occurring complex as above, when present in its natural living host, is not isolated, but the same complex, separated from some or all of the coexisting materials in the natural system, is isolated. Such complexes could be  
25   part of a composition, and still be isolated in that such composition is not part of its natural environment.

          Another aspect of the invention is a kit, suitable for performing any of the methods (*e.g.*, assays) of the invention. For example, the kit may be suitable for treating  
30   a subject (*e.g.*, a subject suffering from a condition mediated by aberrant expression and/or activity of AM or GRP), or for detecting an AM or GRP peptide, *in vitro* or *in*

*vivo*. The components of the kit will vary according to which method is being performed. Generally, a kit of the invention comprises one or more of the compounds of formula I through formula XVII (or, more particularly, I' through XVII'), or a pharmaceutical composition comprising said compound(s) and a pharmaceutically acceptable carrier.

5 The kits also optionally contain means (*e.g.*, suitable reagents) for monitoring disease conditions and/or for detecting AM or GRP. Reagents for performing suitable controls may also be included.

Optionally, the kits comprise instructions for performing the method. Kits of the invention may further comprise a support on which a cell can be propagated (*e.g.*, a tissue  
10 culture vessel) or a support to which a reagent used in the method is immobilized. Other optional elements of a kit of the invention include suitable buffers, media components, or the like; a computer or computer-readable medium for storing and/or evaluating the assay results; logical instructions for practicing the methods described herein; logical  
15 instructions for analyzing and/or evaluating the assay results as generated by the methods herein; containers; or packaging materials. The reagents of the kit may be in containers in which the reagents are stable, *e.g.*, in lyophilized form or stabilized liquids. The reagents may also be in single use form, *e.g.*, in single dosage form for use as therapeutics, or in single reaction form for diagnostic use.

Kits of the invention have many uses, which will be evident to the skilled worker.  
20 For example, they can be used in experiments to study factors involved receptor-mediated activities; to detect the presence of AM or GRP in a cell or tissue, *in vitro* or *in vivo*; to treat a condition mediated by aberrant expression and/or activity of AM or GRP; to monitor the course of such a treatment; or to identify more effective modulatory agents for AM or GRP. A modulatory agent of interest can be characterized by performing  
25 assays with the kit, and comparing the results to those obtained with known agents (or by comparison to a reference). Such assays are useful commercially, *e.g.*, in high-throughput drug studies.

In the foregoing and in the following examples, all temperatures are set forth  
30 uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

## EXAMPLES

### **Example 1 - Small molecule library**

The small molecule repository that the NCI has collected since 1955 was used. This library contains about 500,000 compounds organized in 2,000 families of chemically similar molecules. The construction of the library has been described in Voigt *et al. (supra)* and can be viewed at the web site [cactus.nci.nih.gov/ncbidb2](http://cactus.nci.nih.gov/ncbidb2). All compounds were provided diluted in DMSO.

### **Example 2 – Reagents**

Synthetic human AM and GRP were purchased from Peninsula (S. Carlos, California). Synthetic CGRP and forskolin were obtained from Sigma (St. Louis, Missouri). Blocking monoclonal antibodies against AM<sup>30</sup> and GRP<sup>20</sup> were produced in-house and labeled with peroxidase using EZ-Link Plus Activated Peroxidase (Pierce, Rockford, Illinois).

### **Example 3 – Primary screening for AM and GRP (step #1 of the assay)**

Human synthetic AM was solid-phased into PVC 96-well plates (Fisher Scientific, Pittsburgh, Pennsylvania) by incubating 50 µl of AM (at 1 nmols/µl) per well for 1 h. To solid-phase GRP into the plates, these were previously treated with glutaraldehyde as described (Kasprzyk *et al. (1988) Anal. Biochem.* 174, 224-234). After discarding the coating solution, the plates were blocked with 200 µl per well of 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS). After 1 h, this solution was aspirated off and 50 µl containing 1 µM of one of the compounds of the library in PBS was added per well. Immediately after, 50 µl of labeled antibody (at 2.4 µg/ml) were added on each well and the solution was allowed to react for 1 h. Following 3 thorough washes with 1% BSA in PBS to remove the unbound antibodies, peroxidase activity was developed using o-phenylenediamine dihydrochloride (Sigma) as a substrate. The reaction product was quantified with a plate reader (Spectra Rainbow, Tecan, Austria) at 450 nm. Each plate contained several internal controls including wells without any coating that are used to calculate non-specific binding; wells where no

potential antagonists were added, which provided maximum binding; and wells where the unlabeled antibody (at 1.2 µg/ml) substituted the small molecule, as a positive inhibition control (Fig. 1B). Each compound was added to duplicate wells in the same plate. A positive hit was defined as a compound that was able to significantly reduce the amount of reaction product in three independent plates.

**Example 4 – Analysis of second messengers (step #2 of the assay)**

***a. cAMP analysis for AM and CGRP***

The fibroblast cell line Rat2 has been shown to contain specific AM receptors and react to AM addition by elevating its intracellular cAMP contents. This cell line was obtained from the American Tissue Culture Collection (ATCC, Manassas, Virginia) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, California). Cells were seeded in 24-well plates at  $2 \times 10^4$  cells/well and incubated at 37 °C in 5% CO<sub>2</sub> until they reached 80% confluency. Before the assay, cells were incubated for 15 min in TIS medium (RPMI-1640 plus 10 µg/ml transferrin, 10 µg/ml insulin, and 50 nM sodium selenite) containing 1% BSA, 1 mg/ml bacitracin, and 100 µM isobutylmethylxanthine. Peptides and small molecules were applied in the same medium for 5 min at the indicated concentrations in a volume of 250 µl. The reaction was terminated by adding an equal volume of ice-cold ethanol. cAMP contents were measured using the Biotrac cAMP radioimmunoassay (Amersham Biosciences, Piscataway, New Jersey), as described (Pio *et al.* (2001) *J. Biol. Chem.* 276, 12292-12300).

A cell line expressing the CGRP receptor was generated by transfecting HEK 293 cells with CRLR and RAMP1 (a generous gift from Dr Debbie Hay, Hammersmith Hospital, London, UK). The analysis was performed as above, but using CGRP instead of AM as the main agonist. In both cases, forskolin was used as a positive control at 50 µM.

Details of the above analyses are discussed in the Brief Description of Figure 2, and results of the analyses are shown in Figure 2.



b.  $IP_3$  and  $Ca^{2+}$  analysis for GRP

The lung cancer cell line H-1299 has been shown to contain specific GRP receptors. This cell line was obtained from ATCC and cultured as the other cell lines. The signal transduction pathway for GRP includes elevation of intracellular levels of  $IP_3$  and  $Ca^{2+}$  and these were investigated as previously shown (Ryan *et al.* (1998) *J. Biol. Chem.*, 273, 13613-13624). Briefly, to quantify  $IP_3$ , contents cells were subcultured into 24-well plates ( $5 \times 10^4$  cells/well). After a 24 h incubation period at 37 °C, the cells were incubated with 3  $\mu$ Ci/ml myo- $[^3H]$ inositol in growth medium supplemented with 2% FBS for an additional 24 h. Incubation volumes were 500  $\mu$ l of assay buffer/well containing 135 mM sodium chloride, 20 mM HEPES (pH 7.4), 2 mM calcium chloride, 1.2 mM magnesium sulfate, 1 mM EGTA, 20 mM lithium chloride, 11.1 mM glucose, and 0.05% BSA (v/v) with or without any of the molecules studied at 37 °C for 30 min. Experiments were terminated with 1 ml of ice-cold hydrochloric acid/methanol (0.1%, v/v).  $[^3H]IP_3$  was eluted off Dowex AG-1-X8 anion exchange columns with 2 ml of 1 mM ammonium formate and 100 mM formic acid. Each of the eluates was collected and mixed with 10 ml of scintillation mixture (BioSafe, Research Products International Corp, Mount Prospect, Illinois), and the radioactivity was measured in a LS 3801  $\beta$  counter (Beckman, Somerset, New Jersey).

Calcium levels were analyzed by loading the cells with 2  $\mu$ M FURA-2/AM (Molecular Probes, Eugene, Oregon) for 30 min at 37 °C. After washing two times with TIS, 2 ml of cell suspension were placed in a Delta PTI Scan 1 spectrofluorimeter (Photon Technology International, South Brunswick, New Jersey) equipped with a stir bar and water bath (37 °C). Fluorescence was measured at dual excitation wavelengths of 340 nm and 380 nm using an emission wavelength of 510 nm.

Details of the above analyses are discussed in the Brief Description of Figure 3, and results of the analyses are shown in Figure 3.

**Example 5 – Measurement of blood pressure *in vivo***

AM is a potent and long-lasting vasodilator. Therefore it was expected that AM antagonists would elevate blood pressure and AM superagonists would decrease it further. In consequence, suspected antagonists were analyzed in normotensive rats (10-

week-old Lewis/ssnrc males, SAIC, Frederick, Maryland) and suspected superagonists in hypertensive animals (10-week-old SHR males, Taconic Farms, Germantown, NY).

Animals were anaesthetized with 3% halothane, intubated, and maintained with 1% halothane in 70% nitrous oxide and 30% oxygen (VMS Anesthesia Machine, Matrx, Medical Inc., Orchard Park, New York) at 82 strokes/min. Rectal temperature was monitored through the experiment. A PE50 catheter was placed on the right femoral artery and arterial blood pressure was recorded through a P23XL transducer (Grass Instruments, Quincy, Massachusetts). Peptides and small molecules were injected into the right femoral vein through another catheter. All procedures were performed under a protocol approved by the National Institutes of Health.

Injection of screen selected positive modulators of AM (at 20 nmols/Kg) in hypertensive rats induced a long-lasting decrease in blood pressure (Figures 4A and 4B), when compared to basal levels. These differences were  $62 \pm 21$  Hg ( $p < 0.05$ ) for compound 128911 and  $55 \pm 24$  mm Hg ( $p < 0.05$ ) for 145425. Vehicle alone (DMSO in PBS) at the same concentration did not alter blood pressure (Figure 4A). On the other hand, when screen selected negative modulators of AM were injected into normotensive animals, also at 20 nmols/Kg, an elevation in blood pressure was observed (Figure 4C). In the case of compound 16311, the difference from basal levels was  $127 \pm 47$  mm Hg ( $p < 0.01$ ). These data are shown in Figure 4.

#### **Example 6 - Cord formation assay**

Formation of tube-like structures was performed as described in Kubota *et al.* (1988) *J. Cell Biol.* **107**, 1589-1598; Nam *et al.* (2003) *Phytother. Res.* **17**, 107-111; and Macpherson *et al.* (2003) *Mol Cancer Ther* **2**, 845-54. Briefly, a thin layer of Matrigel (Collaborative Biomedical Products, Bedford, Massachusetts) was allowed to polymerize at the bottom of 24-well plates. Bovine retinal microvascular endothelial cells (a gift from Dr Patricia Becerra, NEI, NIH) were resuspended in Human Endothelial-SFM Basal Growth Medium (Invitrogen) and applied to triplicate wells ( $2 \times 10^5$  cells/500  $\mu$ l medium) in the presence or absence of the test compounds. After an overnight incubation at 37 °C, the tubular structures were photographed (3 pictures per well at 10x)

and the number of knots per photographic field were counted as a measure of lattice complexity.

As shown in Figure 5, GRP (5 nM) was able to induce cord formation in a culture of endothelial cells grown on Matrigel (Fig. 5A,B) whereas the addition of 0.5  $\mu$ M of the screen identified compound XV' (77427) greatly reduced the complexity of the tubular lattice (Fig. 5C). The number of knots per photographic field went from  $3 \pm 1$  (control) to  $37 \pm 5$  for the addition of 5nM GRP ( $p < 0.001$ ) and back to  $12 \pm 4$  when GRP and 77427 were added together (compared to control  $p = 0.02$ , compared to GRP alone  $p = 0.003$ ).

#### **Example 7 - Directed *in vivo* angiogenesis assay (DIVAA)**

Analysis and quantitation of angiogenesis was done using DIVAA as previously described (Martinez *et al.* (2002) *J Natl Cancer Inst* 94, 1226-37). Briefly, 10 mm long surgical-grade silicone tubes with only one end open (angioreactors) were filled with 20  $\mu$ l of matrigel alone or mixed with GRP and the small molecules at the indicated concentrations. After the matrigel solidified, the angioreactors were implanted into the dorsal flanks of athymic nude mice (NCI colony). After 11 days, the mice were injected iv with 25 mg/ml FITC-dextran (100  $\mu$ l/mouse, Sigma) 20 min before removing angioreactors. Quantitation of neovascularization in the angioreactors was determined as the amount of fluorescence trapped in the implants and was measured in a HP Spectrophotometer (Perkin Elmer).

As shown in Figure 6, 1 nM GRP exhibits angiogenic potential *in vivo*; and both the small molecule antagonist, compound XV' (77427), and the anti-GRP monoclonal antibody, 2A11, exhibit a dose-dependent inhibition of GRP-induced angiogenesis.

#### **Example 8 - Proliferation assays**

A. Tumor cells (from a lung cancer cell line, H1299) were seeded in 96-well plates at a density of  $2.0 \times 10^5$  cells per well in serum-free medium containing different concentrations of the test agent, the small molecule inhibitor compound XV' (77427). After 5 days in culture, the number of viable cells per well was estimated by the growth inhibition MTT assay (as reported in Iwai *et al.* (1999) *Lung Cancer* 23, 209-22).

Figure 7 shows that compound XV' (77427) exhibits a modest dose-dependent growth inhibitory action on the lung cancer cells.

5 B. Cells from the cell line A345 were grown in substrate-independent conditions (clonogenic assay) as previously published (Iwai *et al.*, *supra*).

Figure 8 shows that the small molecule inhibitor compound XV' (77427) reduces the number of colonies developed over a period of 3 weeks in soft agar. (The results are represented as percentage growth over the untreated control.)

10 **Example 9 - Assay of *in vivo* tumor growth (xenograft experiment)**

Thirty female athymic nude mice from the NIH colony in Frederick (MD) were injected subcutaneously with  $1.0 \times 10^7$  H1299 cells/mouse. Two weeks later, all the mice had developed palpable tumors under the skin and at this time they were randomly divided in three groups. Three times a week, each individual tumor was measured (length, height, thickness) and every mouse received an intratumoral injection, according to their group. Group 1 (control) received 100  $\mu$ l PBS (negative control); group 2 received 100  $\mu$ l 0.5  $\mu$ M compound XV' (77427) in PBS; and group 3 received 100  $\mu$ l 5  $\mu$ M compound XV' (77427) in PBS. When the tumor burden became unbearable (larger than 2000 mm<sup>3</sup>), the mice were sacrificed.

20 Figure 9 shows that the tumor size was significantly reduced after a single injection of compound XV', and that the tumors all but disappeared after the second injection.

**Example 10 - Statistics**

25 Different treatments were compared with two-tailed Student's *t* test. P values smaller than 0.05 were considered statistically significant.

**Example 11 - Characterization of the binding between small molecules and AM**

**A. Methods**

30 **Surface Plasmon Resonance Assays**

Characterization of the binding between AM and the small molecules was performed immobilizing 3  $\mu$ g of AM on a CM5 sensor chip by N-hydroxysuccinimide activation, followed by covalent amino coupling of the peptide to the surface, using BIAcore 3000 (Piscataway, NJ). The remaining free surface was blocked with 1 mM ethanolamine and the matrix washed with 0.5 M NaCl solution and then re-equilibrated with binding buffer (1:200 DMSO in PBS). Eight different dilutions of each small molecule were prepared in binding buffer with concentrations ranging from 0 to 10  $\mu$ M and injected from low to high concentration. Each injection was followed by a matrix regeneration step. Mass transfer control experiments were performed by injecting the same concentration of each small molecule at different flow rates (5, 15, and 75  $\mu$ l/min). The data were then fitted to several models for a kinetic analysis and the binding constants calculated. The best fittings were obtained with a simple 1:1 Langmuir model.

#### Receptor binding assays

Binding of  $^{125}$ I-AM to Rat2 cells was performed as described in Martinez *et al.* (1997) *Endocrinology* 138, 5597-5604. Briefly,  $5 \times 10^4$  cells were placed in 24-well plates coated with fibronectin (20  $\mu$ g/well). When a monolayer was formed, the cell were washed 3 times in transferrin, insulin, and selenium (TIS) medium, followed by incubation with receptor-binding medium (TIS plus 1% BSA and 1 mg/ml bacitracin) with 0.2 nM  $^{125}$ I-AM (2200 Ci/mmol, Phoenix) in the presence or absence of competitors (cold AM or small molecules). After 2 h at 4°C, free peptide was removed by washing 3 times in receptor-binding medium. Peptide bound to the cells was solubilized in 0.2 N NaOH and counted in a  $\gamma$ -counter.

#### B. Results - Characterization of the binding between the small molecules and AM

To determine the mechanism of action of the small molecules, we first performed receptor binding assays and saw no change in the affinity of AM for its receptor in the presence or absence of the small molecule regulators, indicating that these molecules are not receptor modulators. The other possibility is a direct binding to the peptide. This was demonstrated by surface plasmon resonance assays. AM was immobilized into the chip's gold surface and the binding of the small molecules was followed by their effect on the angle of the reflected light. This effect was dose-dependent, allowing for a kinetic analysis of the binding. The calculated KDs varied from  $2.93 \times 10^{-6}$  for compound

128911 to  $6.56 \times 10^{-9}$  for compound 16311. One of the molecules identified for its binding to GRP (54671) was used as a control and was shown not to bind to immobilized AM. A summary of the results is presented in Table 2:

Analytes	Peptide target	Response to second messengers	Biological activity	Binding to AM			
				ka (1/Ms)	kd (1/s)	KA (1/M)	KD (M)
89435	AM	Neg. modul.	Vasodilator	$1.02 \times 10^3$	$8.71 \times 10^{-4}$	$1.18 \times 10^6$	$8.51 \times 10^{-7}$
16311	AM	Neg. modul.	Vasodilator	$1.55 \times 10^3$	$1.02 \times 10^{-5}$	$1.52 \times 10^8$	$6.56 \times 10^{-9}$
128911	AM	Posit. modul.	Vasopressor	304	$8.92 \times 10^{-4}$	$3.41 \times 10^3$	$2.93 \times 10^{-6}$
54671	GRP	Neg. modul.		No binding			

Table 2

Characterization of the binding between a few selected small molecules and AM or GRP. ka: kinetic association constant. kd: kinetic dissociation constant. KA: thermodynamic association constant. KD: thermodynamic dissociation constant.

To examine a potential mass transfer influence, a constant concentration of the small molecule was injected and allowed to react with the immobilized peptide at different flow rates. These experiments clearly demonstrate that binding rates are independent of flow rate and a mass transfer influence could therefore be ruled out.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire disclosure of all applications, patents and publications, cited above and below and in the figures are hereby incorporated by reference.

### References

- US patent applications 20020019347, 20020055615 (Cuttitta *et al.*) and 20020176819;
- 5    USPs 5,109,115 (Cuttitta), 5,460,801 (Cuttitta), 4,517,188, 5,834,433, 5,047,502, and 5,620,955; EP 806418; Japanese patent JP 10212235; Isumi *et al.* (1998) *Endocrinology* 139, 2552-2563; Coppock *et al.* (1999) *Biochem J.* 338, 15-22; Ishizaka *et al.* (1994) *Biochem. Biophys. Res. Comm.* 200, 642-646; Heimbrook *et al.* (1991) *J. Med. Chem.* 34, 2102-2107; O'Brien *et al.* (1965) *Journal of Medicinal Chemistry* 8, 182-7; Pficiderer *et*
- 10   *al.* (1961) *Chemische Berichte* 94, 2708-21; Gibson *et al.* (1998) *Journal of the American Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry* 18, 3025-30325; Schally *et al.* (2001) *Front. Neuroendocrinol.* 22, 248-91; Bajo *et al.* (2004) *Br. Journal of Cancer* 90, 245-252; Ganguly *et al.* (1963) *Indian Journal of Chemistry* 1(8), 364; Draoui, Dissertation, George Washington University, 1993.

15

WE CLAIM:

1. A complex comprising a compound of one of formula I - VIII, XII or XIII, in association with an adrenomedullin (AM) peptide.

2. The complex of claim 1, wherein the compound is selected from formula I' - XIII'.

3. The complex of claim 1, which is in an animal.

4. The complex of claim 1, which is *in vitro*.

5. A complex comprising a compound of one of formula XIV, XVI or XVII, in association with a gastric releasing peptide (GRP).

6. The complex of claim 4, wherein the compound is of one of formula XIV', XVI' or XVII'.

7. The complex of claim 4, which is in an animal (*e.g.*, a mammal).

8. The complex of claim 4, which is *in vitro*.

9. A pharmaceutical composition comprising a compound of one of formula I - VIII, XII - XIV, or XVI - XVII, and a pharmaceutically acceptable carrier.

10. The pharmaceutical composition of claim 9, wherein the compound is of one of formula I' - XIV' or XVI' - XVII'.

11. A method for inhibiting an activity of an AM peptide, comprising contacting the peptide with an effective amount of a compound of one of formula I - VII.

12. The method of claim 11, wherein the compound is of one of formula I' - VII'.



13. The method of claim 11, wherein the peptide and compound are in an animal.
14. The method of claim 11, wherein the peptide and compound are *in vitro*.
- 5 15. The method of claim 11, wherein the activity of the AM peptide is stimulation of the level of intracellular cAMP.
16. The method of claim 11, wherein the activity of the AM peptide is vasodilation.
- 10 17. A method for treating a condition that is mediated by over-expression and/or activity of AM, comprising administering to a patient in need of such treatment an effective amount of a compound of one of formula I - VII.
18. The method of claim 17, wherein the compound is of one of formula I' - VII'.
- 15 19. The method of claim 17, wherein the condition is type 2 diabetes or cancer.
20. A method for stimulating an activity of an AM peptide, comprising contacting the peptide with an effective amount of a compound of one of formula VIII, XII or XIII.
- 20 21. The method of claim 20, wherein the compound is of one of formula VIII' - XIII'.
22. The method of claim 20, wherein the peptide and compound are in an animal.
- 25 23. The method of claim 20, wherein the peptide and compound are *in vitro*.
24. The method of claim 20, wherein the activity of the AM peptide is stimulating the level of intracellular cAMP.
- 30 25. The method of claim 20, wherein the activity of the AM peptide is vasodilation.

26. A method for treating a condition that is mediated by under-expression and/or activity of AM, comprising administering to a patient in need of such treatment an effective amount of a compound of one of formula VIII, XII or XIII.

5 27. The method of claim 26, wherein the compound is of one of formula VIII' - XIII'.

28. The method of claim 26, wherein the condition is renal or cardiovascular disease, sepsis, or central nervous system ischemia.

10 29. A method for inhibiting an activity of a GRP peptide, comprising contacting the peptide with an effective amount of a compound of formula XIV or XVI.

30. The method of claim 29, wherein the compound is of formula XIV' or XVI'.

15 31. The method of claim 29, wherein the peptide and compound are in an animal.

32. The method of claim 29, wherein the peptide and compound are *in vitro*.

20 33. The method of claim 29, wherein the activity of the GRP peptide is the stimulation of intracellular IP<sub>3</sub> or Ca<sup>+2</sup>.

34. The method of claim 29, wherein the activity of GRP is stimulating angiogenesis, suppressing food intake, regulating glucose homeostasis, or stimulating hypotension.

25 35. A method for treating a condition that is mediated by over-expression and/or activity of GRP, comprising administering to a patient in need of such treatment an effective amount of a compound of formula XIV or XVI.

30 36. The method of claim 35, wherein the compound is of formula XIV' or XVI'.

37. The method of claim 35, which is a method for inhibiting or reducing tumor growth, treating low blood pressure or an eating disorder, or improving breathing in a premature baby.

5 38. A method for stimulating an activity of a GRP peptide, comprising contacting the peptide with an effective amount of a compound of formula XVII.

39. The method of claim 38, wherein the compound is of formula XVII'.

10 40. The method of claim 38, wherein the peptide and compound are in an animal.

41. The method of claim 38, wherein the peptide and compound are *in vitro*.

15 42. The method of claim 38, wherein the activity of the GRP peptide is the stimulation of intracellular IP<sub>3</sub> or Ca<sup>+2</sup>.

43. The method of claim 38, wherein the activity of GRP is stimulating angiogenesis, suppressing food intake, regulating glucose homeostasis, or stimulating hypotension.

20 44. A method for treating a condition that is mediated by under-expression and/or activity of GRP, and/or that would benefit from increased expression of GRP comprising administering to a patient in need of such treatment an effective amount of a compound of formula XVII.

25 45. The method of claim 44, wherein the compound is of formula XVII'.

46. The method of claim 44, wherein in the condition is obesity, diabetes, hypertension, coronary or peripheral artery disease, tissue ischemia, organ or tissue transplantation, or acceleration or enhancing of fracture repair or wound healing.

30 47. A method for detecting an AM peptide, comprising

contacting a sample suspected of containing the peptide with one or more detectably labeled compounds of formula I through VIII, or formula XII through XIII, and

detecting labeled compound that is associated with the peptide.

5

48. The method of claim 38, wherein the detectably labeled compound is of formula I' - XIII'.

49. A method for detecting a GRP peptide, comprising

10

contacting a sample suspected of comprising the peptide with one or more detectably labeled compounds of formula XIV - XVII, and  
detecting labeled compound that is associated with the peptide.

50. The method of claim 49, wherein the detectably labeled compound is selected from  
15 formula XIV' through XVII'.

51. The method of claim 47 or 49, which is performed *in vivo*.

52. The method of claim 47 or 49, which is performed *in vitro*.

20

53. A kit suitable for treating a subject suffering from a condition mediated by aberrant expression and/or activity of adrenomedullin (AM), comprising one or more compounds of formula I - VIII, XII or XIII, or a pharmaceutical composition comprising said compound(s) and a pharmaceutically acceptable carrier, and, optionally, a container or  
25 packaging material.

54. The kit of claim 53, wherein the compound(s) is selected from formula I' through XIII'.

30

55. A kit suitable for treating a subject suffering from a condition mediated by an aberrant expression and/or activity of gastrin releasing peptide (GRP), comprising one or

more of the compounds of formula XIV, XVI or XVII, or a pharmaceutical composition comprising said compound(s) and a pharmaceutically acceptable carrier, and, optionally, a container or packaging material.

5 56. The kit of claim 55, wherein the compound(s) is of one of formula XIV', XVI' and/or XVII'.

57. A kit suitable for detecting an AM peptide, comprising

- 10 a) one or more compounds selected from formula I - VIII, XII or XIII, which is detectably labeled, and, optionally,  
b) means to detect the labeled compound associated with (bound to) the peptide.

58. The kit of claim 57, wherein the compound(s) is of one of formula I' - XIII'.

15 59. A kit suitable for detecting a GRP peptide, comprising

- a) one or more compounds selected from formula XIV - XVII, which is detectably labeled, and, optionally,  
b) means to detect the labeled compound associated with (bound to) the peptide.

20 60. The kit of claim 45, wherein the compound(s) is selected from formula XIV' - XVII'.

61. A kit of claim 53 or 55, which is suitable *in vivo* detection, further wherein said compound is in a pharmaceutically acceptable carrier.

25 62. A method for inhibiting GRP-mediated angiogenesis in a subject in need of such treatment, comprising administering to the subject an effective amount of an agent that inhibits GRP,  
provided that the GRP-mediated angiogenesis is not angiogenesis involved in tumor growth or metastasis.

30

63. A method for preventing or treating condition mediated by GRP-mediated angiogenesis in a subject in need of such treatment, comprising administering to the subject an effective amount of an agent that inhibits GRP,  
provided that the condition is not cancer.

64. A method for preventing or treating one of the following angiogenesis-mediated conditions in a subject:

arthritis (*e.g.*, rheumatoid arthritis),

psoriasis,

benign growths caused by rapidly dividing cells (*e.g.*, noncancerous melanomas),  
brain ischaemia,

vascular diseases (*e.g.* atherosclerosis, myocardial angiogenesis, post-balloon angioplasty vascular restenosis, neointima formation following vascular trauma, vascular graft restenosis, coronary collateral formation, deep venous thrombosis, ischemic limb angiogenesis),

ocular diseases involving ocular neovascularization or related ocular diseases and disorders (*e.g.*, diabetic neovascularization, neovascular glaucoma, macular degeneration, diabetic and other retinopathy, retrolental fibroplasia and corneal diseases),

fibrosis (*e.g.*, fibrosis associated with a chronic inflammatory condition, lung fibrosis, chemotherapy-induced fibrosis, wound healing (*e.g.*, of chronic wounds) with scarring and fibrosis),

deep venous thrombosis,

endometriosis,

wrinkles, etc,

comprising administering to the subject an effective amount of an agent that inhibits GRP.

65. A method for inhibiting angiogenesis-mediated tumor growth in a subject in need of such treatment, comprising

administering to the subject an effective amount of an agent that inhibits GRP,  
and

detecting or monitoring the reduction in blood vessels (inhibition of angiogenesis).

66. The method of claim 62, wherein the agent is a compound of formula XV.

5

67. The method of claim 63, wherein the agent is a compound of formula XV.

68. The method of claim 64, wherein the agent is a compound of formula XV.

10

69. The method of claim 65, wherein the agent is a compound of formula XV.

70. The method of claim 62, wherein the agent is a compound of formula XV'.

71. The method of claim 63, wherein the agent is a compound of formula XV'.

15

72. The method of claim 64, wherein the agent is a compound of formula XV'.

73. The method of claim 65, wherein the agent is a compound of formula XV'.

74. A method for treating low blood pressure or an eating disorder in a subject in need of such treatment, comprising administering to the subject an effective amount of a compound of formula XV.

20

75. The method of claim 74, wherein the compound is of formula XV'.

## **ABSTRACT OF THE INVENTION**

This invention relates, *e.g.*, to methods for inhibiting or stimulating an activity of an adrenomedullin (AM) or gastrin releasing peptide (GRP) peptide hormone, comprising contacting the peptide with a small molecule, non-peptide, modulatory agent of the  
5 invention. Complexes of these modulatory agents with other components, such as the peptides or blocking antibodies specific for the peptides, are also described, as are pharmaceutical compositions comprising the modulatory agents, and methods for using the modulatory agents to diagnose or treat patients.

10 DOCS #546199



Figure 1

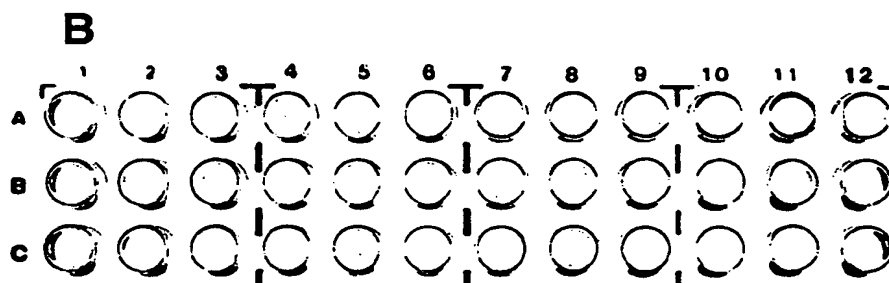
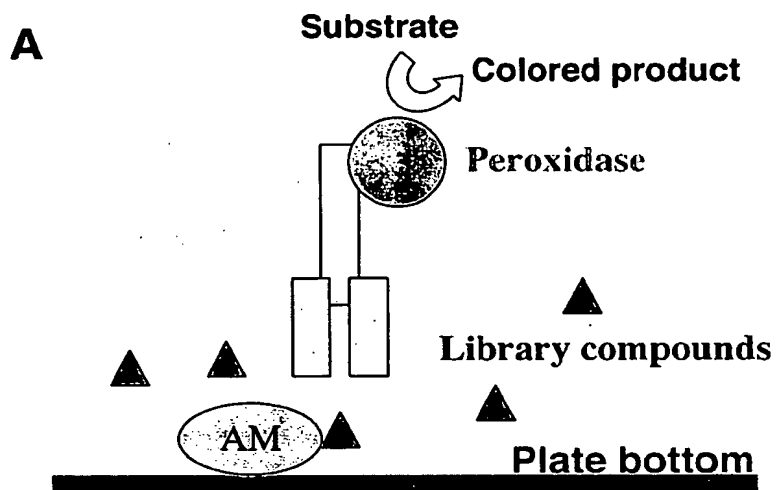


Figure 2

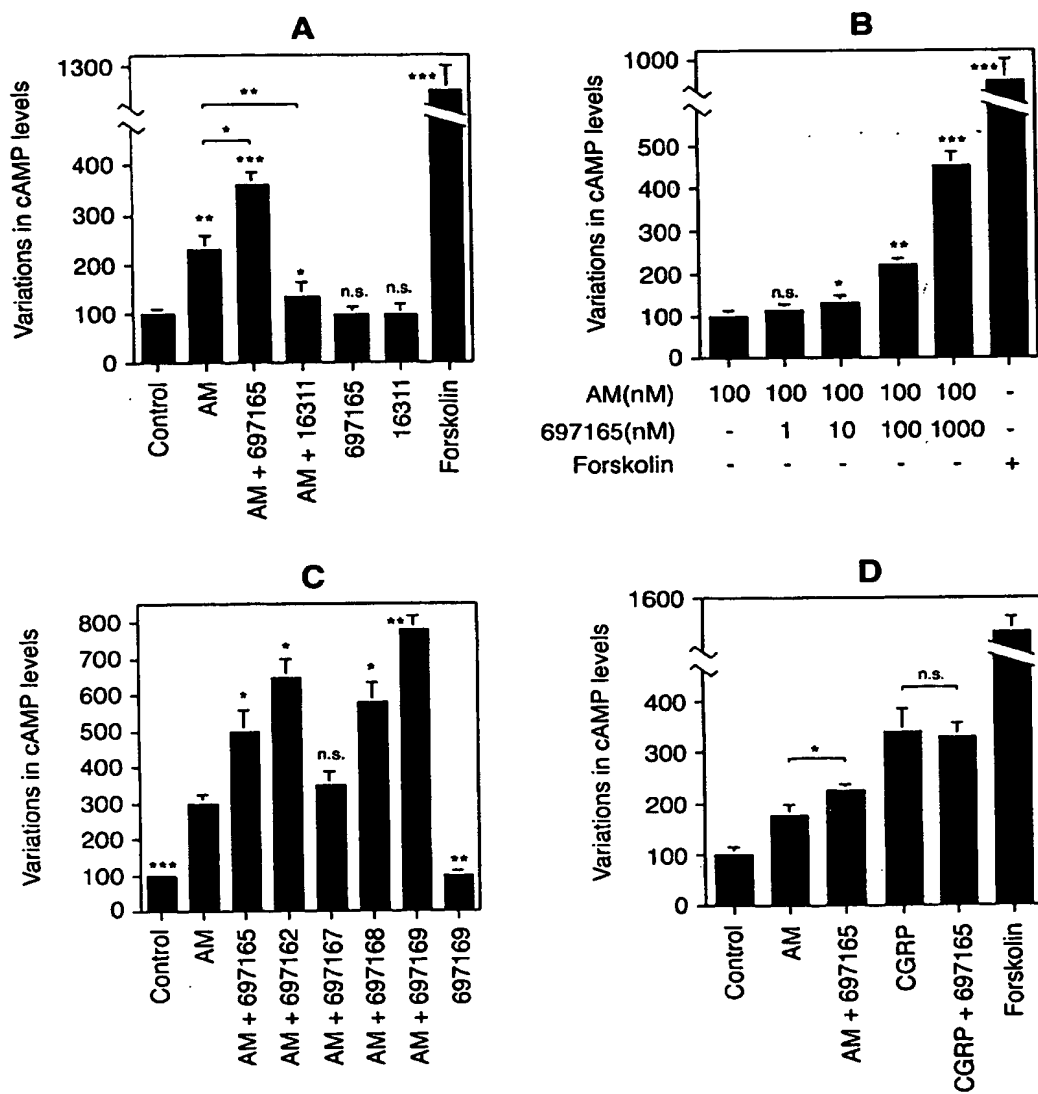


Figure 3

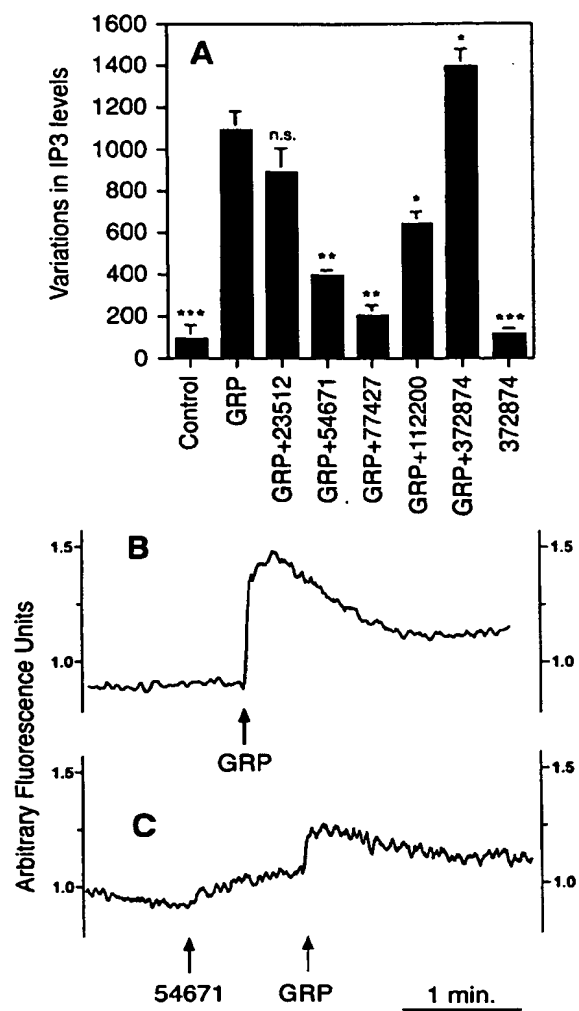
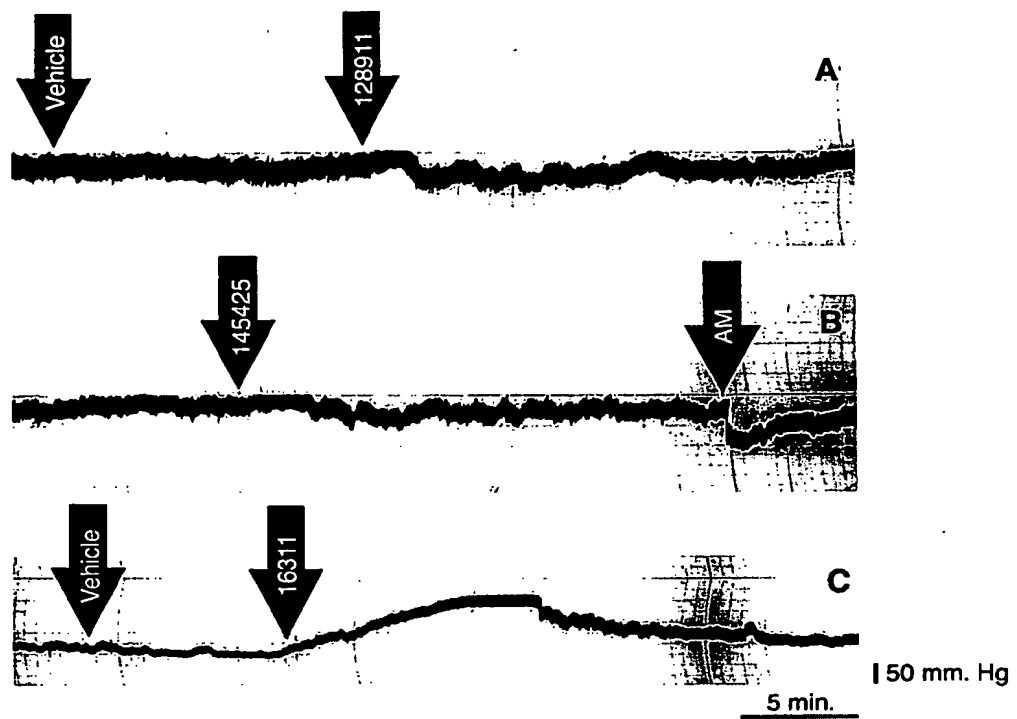
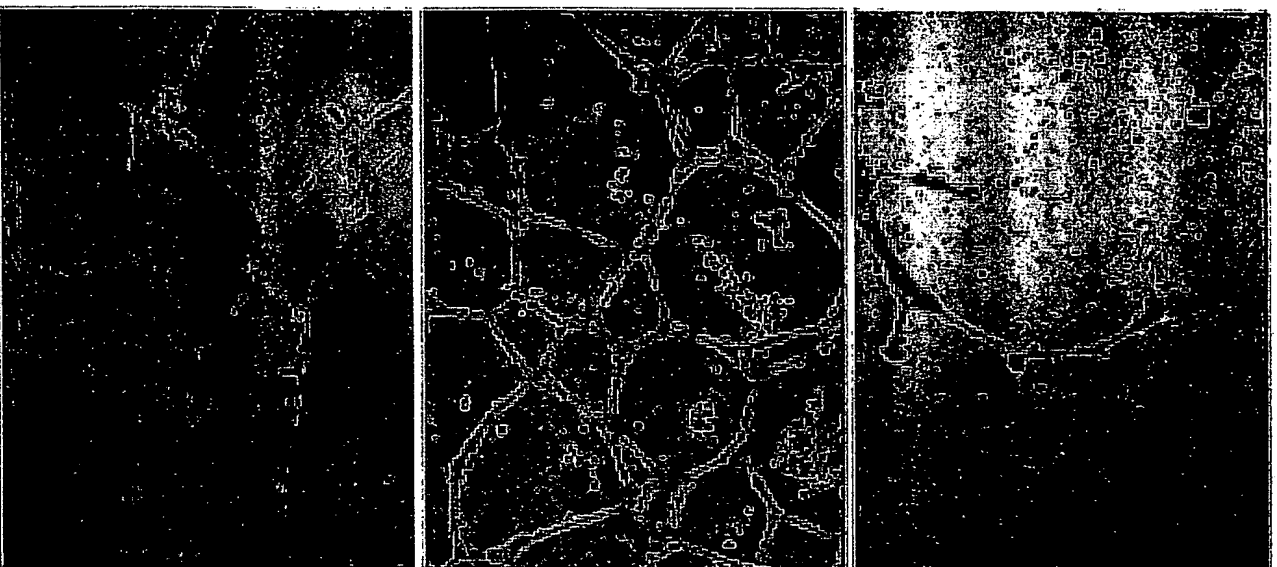


Figure 4





Control

5 nM GRP

5 nM GRP + 0.5  $\mu$ M 77427

Figure 5

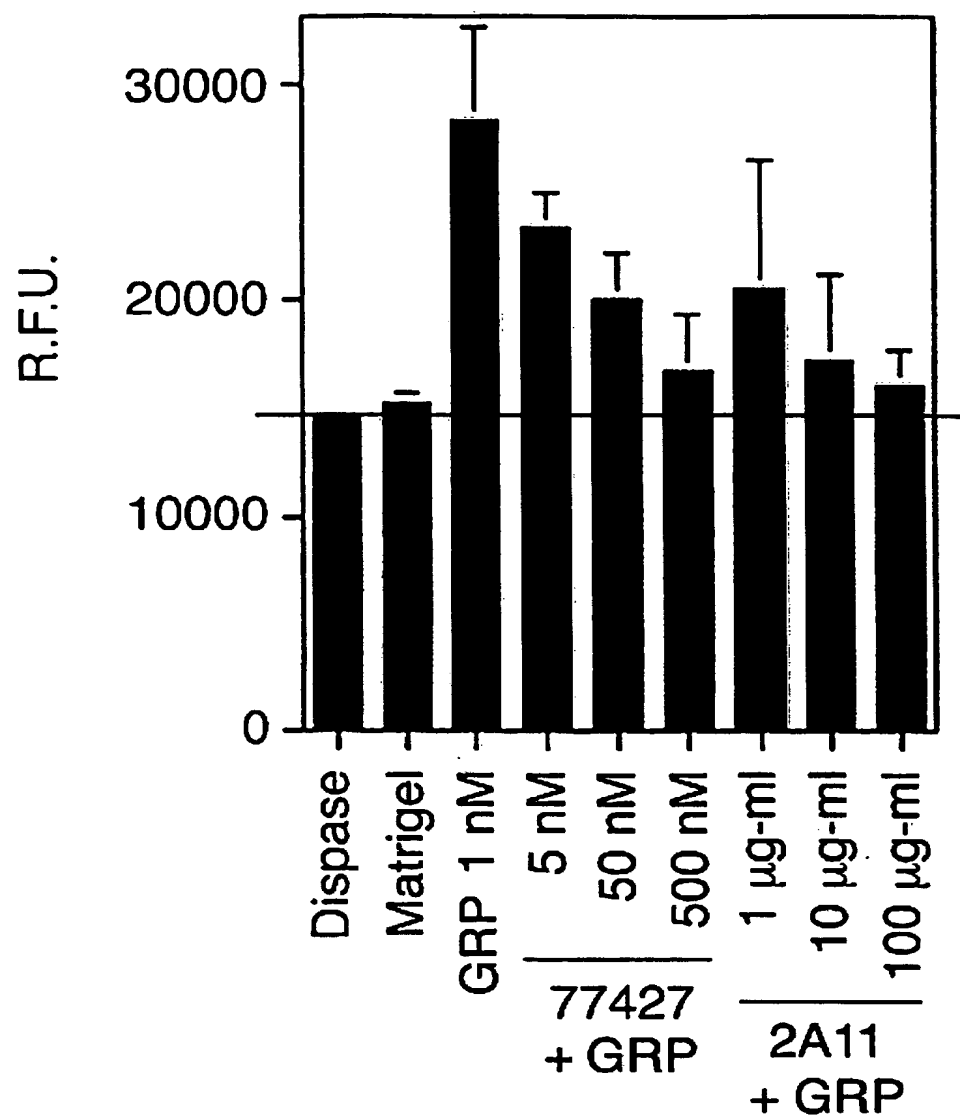
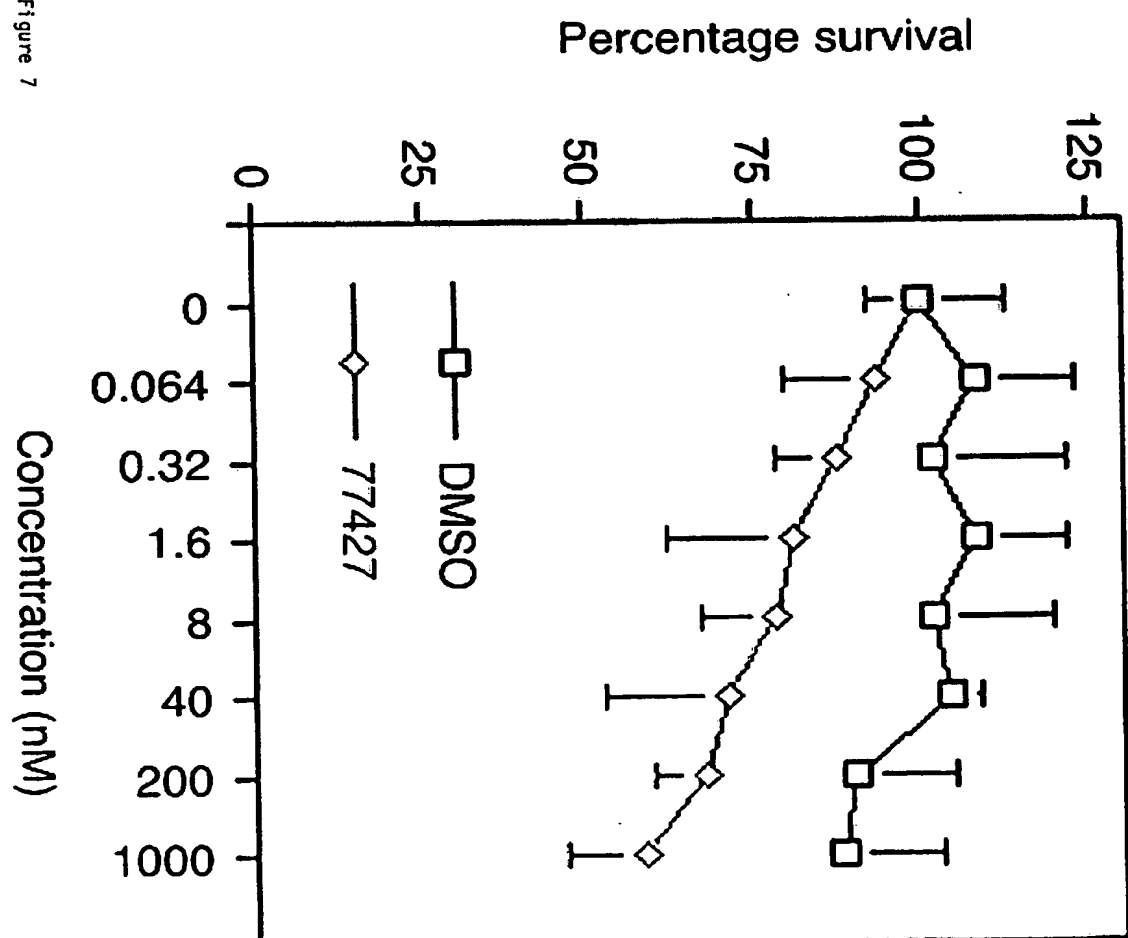


Figure 6

Figure 7



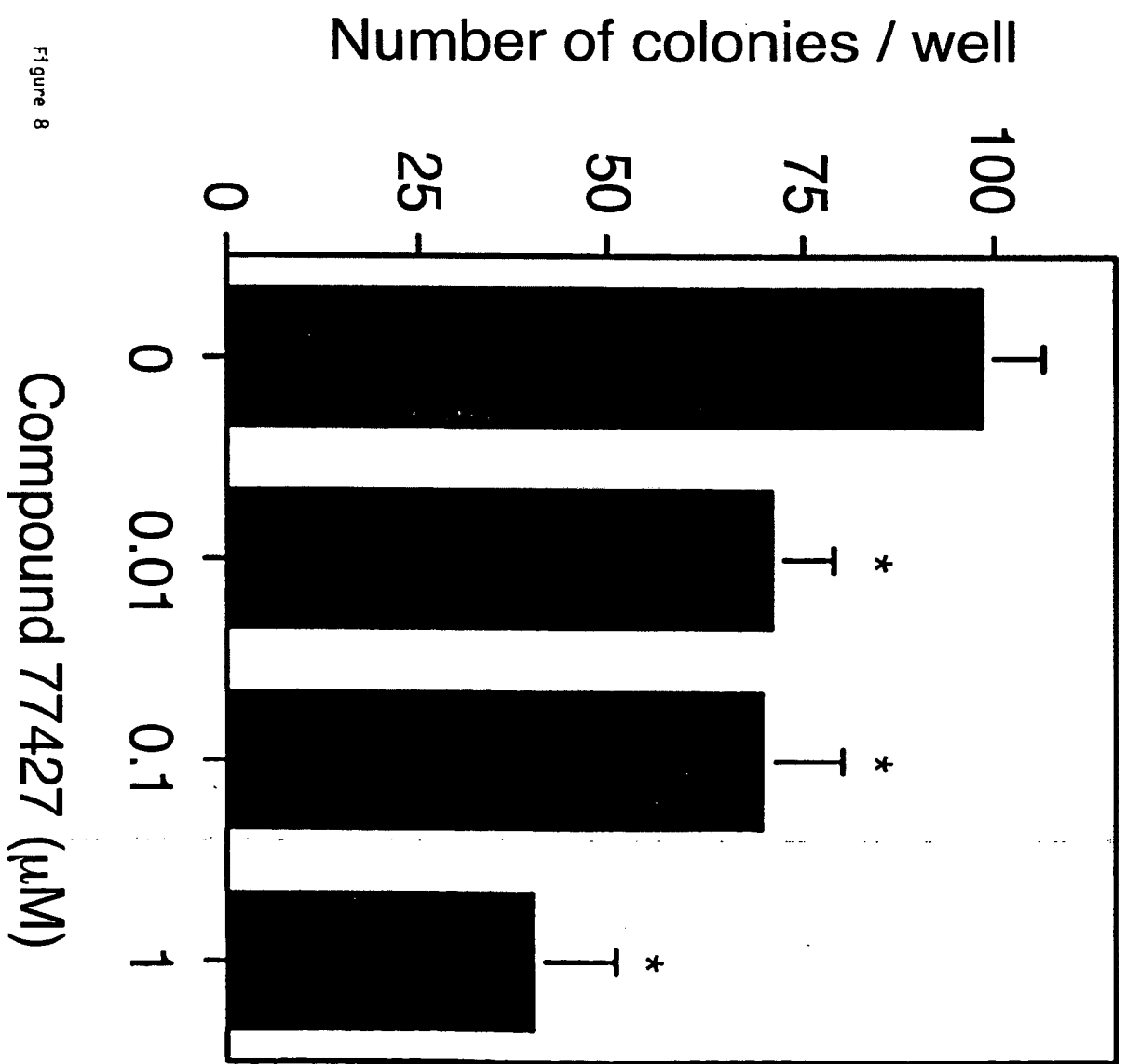


Figure 8



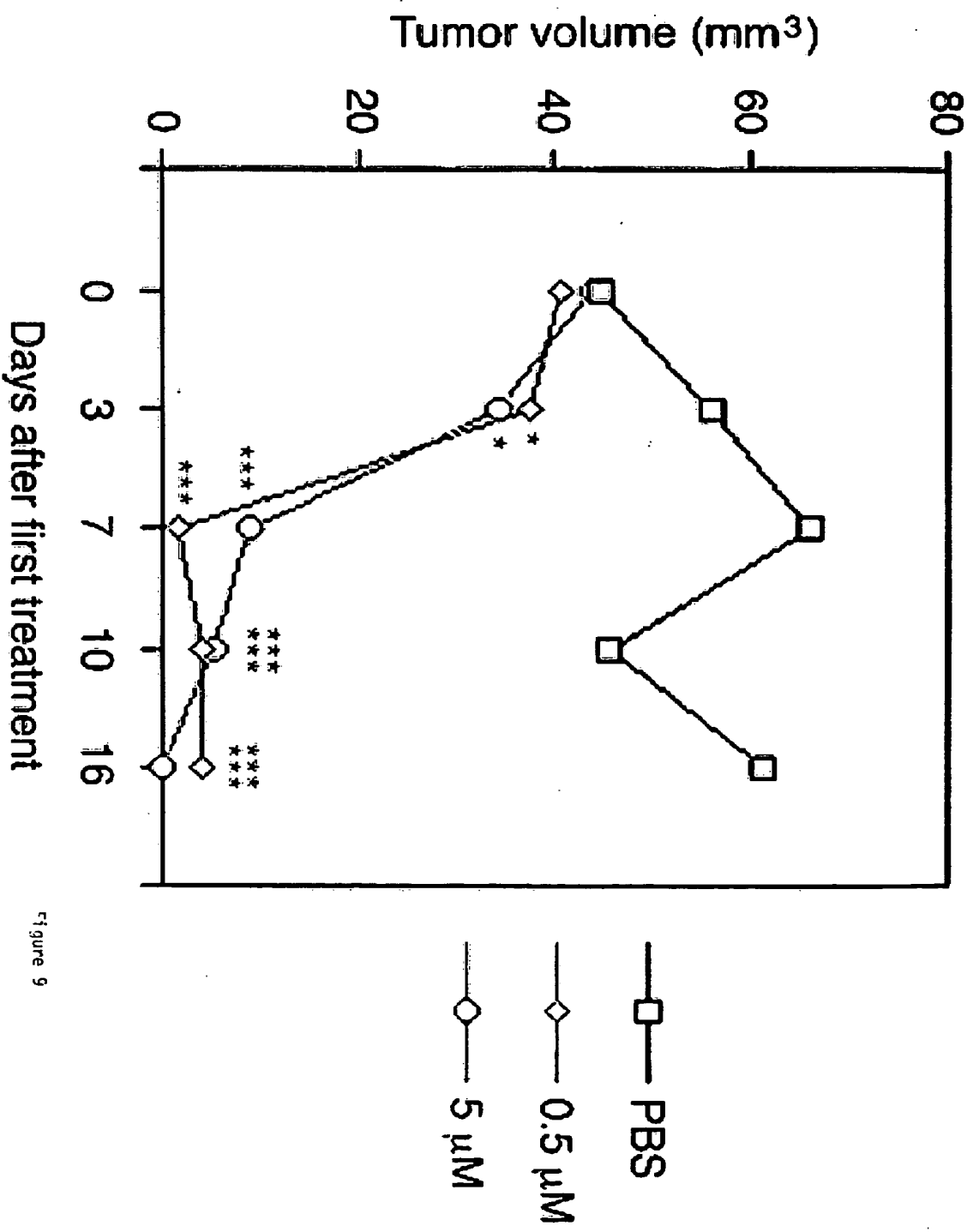


Figure 9

## **APPLICATION DATA SHEET**

### **Application Information**

**Application Number::** To Be Assigned  
**Filing Date::** May 11, 2004  
**Application Type::** Provisional  
**Subject Matter::** Utility  
**Suggested Classification::**  
**Suggested Group Art Unit::**  
**CD-ROM or CD-R?::**  
**Number of CD Disks::**  
**Number of Copies of CDs::**  
**Sequence Submission?::**  
**Computer Readable Form (CFR)?::**  
**Number of Copies of CFR::**  
**Title::** A METHOD FOR INHIBITING ANGIOGENESIS,  
USING A SMALL MOLECULE  
**Attorney Docket Number::** 31978-201011  
**Request for Early Publication?::**  
**Request for Non-Publication?::**  
**Suggested Drawing Figure::**  
**Total Drawing Sheets::** 9  
**Small Entity?::** No  
**Latin Name::**  
**Variety Denomination Name::**  
**Petition Included?::**  
**Petition Type::**  
**Licensed US Govt. Agency::** National Institutes of Health  
**Contract or Grant Numbers::**  
**Secrecy Order in Parent Appl.::**

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**State or Province of Mailing Address::** Maryland  
**Country of Mailing Address::** USA  
**Postal or Zip Code of Mailing Address::** 20814

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**State or Province of Residence::**  
**Country of Residence::**  
**Street of Mailing Address::**  
**City of Mailing Address::**  
**State or Province of Mailing Address::**  
**Country of Mailing Address::**  
**Postal or Zip Code of Mailing Address::**

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**Middle Name::**  
**Family Name::**

**Name Suffix::**

**City of Residence::**

**State or Province of Residence::**

**Country of Residence::**

**Street of Mailing Address::**

**City of Mailing Address::**

**State or Province of Mailing  
Address::**

**Country of Mailing Address::**

**Postal or Zip Code of Mailing  
Address::**

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**E-Mail Address::**

### **Representative Information**

**Representative Customer Number::** 26694

### **Domestic Priority Information**

<b>Application::</b>	<b>Continuity Type::</b>	<b>Parent Application::</b>	<b>Parent Filing Date::</b>
	<b>Continuation of</b>		
	<b>Continuation of</b>		
	<b>Continuation of</b>		
	<b>Continuation of</b>		

## Foreign Priority Information

Country::	Application Number::	Filing Date::	Priority Claimed::

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**Postal or Zip Code of Mailing Address::** 20852-3804

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